Contractor's Final Report 1/
DSS Contract 43SS.KN107-2-4079

ENFOR Project C-181 (2)

Conversion of Cellulose to Ethanol Using a Two-Stage Process

by

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June 1983

TABLE OF CONTENTS

	Page
Summary	v
Introduction	1
Materials and Methods	4
Results	9
Discussion	25
References	32
Tables	
Figures	

List of Tables

Table 1. Location of cellulase enzymes of Trichoderma reesei C30 grown on various substrates after 8 days incubation

Table 2. Location of cellulase enzymes of Trichoderma harzianum E58 grown on various substrates

Table 3. Effect of two concentration techniques on the cellulase enzymes of Trichoderma reesei C30

Table 4. Effect of two concentration techniques on the cellulase enzymes of Trichoderma harzianum E58

Table 5. Storage of concentrated cellulase enzymes from Trichoderma E58

Table 6. Purification of concentrated cellulase enzymes from Trichoderma harzianum E58

Table 7. Temperature profile for enzymatic cellulase assays for Trichoderma harzianum E58

Table 8. Production of reducing sugars and glucose from 5% solka floc under various temperatures of hydrolysis

Table 9. Production of ethanol under various hydrolysis temperatures in a combined hydrolysis and fermentation procedure

Table 10. Production of cellulase enzymes under various media volumes using 2% solka floc

Table 11. Production of cellulase enzymes by Trichoderma harzianum E58 on different growth substrates

Table 12. Production of cellulase enzymes from three different fungi using 5% steam-exploded wood as the substrate

Table 13. Production of cellulase enzymes from three different fungi using 2% steam-exploded wood as the substrate

Table 14. Production of cellulase enzymes from Trichoderma reesei C30 grown on 2% pretreated wood substrate which had been exposed to 20 seconds of steaming

Table 15. Production of cellulase enzymes from Trichoderma harzianum E58 grown on 2% pretreated wood substrates which had been exposed to 20 seconds of steaming

Table 16. Production of cellulase enzymes from Trichoderma reesei C30 grown on 2% pretreated wood substrates which had been exposed to 60 seconds of steaming

Table 17. Production of cellulase enzymes from Trichoderma harzianum E58 grown on 2% pretreated wood substrates which had been exposed to 60 seconds of steaming

Table 18. Production of reducing sugars and glucoses by cellulase enzymes from NOVO and T. harzianum E58

Table 19. Composition of steam-exploded Aspen exposed to 20 seconds steaming and following various extractions

Table 20. Production of reducing sugars and glucose by T. harzianum E58 from various pretreated wood substrates

Table 21. Effect of yeast extract on the hydrolysis of 5% solka floc and the production of ethanol following combined hydrolysis and fermentation

Table 22. Composition of steam-exploded Aspen which was acid-soaked with H_2SO_4 prior to steaming

Table 23. Comparison between effectiveness of extraction procedure with and without acid-soaking prior to steam-explosion

Table 24. Combined hydrolysis and fermentation of wood which was acid soaked prior to steam explosion at 20 seconds of steaming

Table 25. Recovery of sugars following acid hydrolysis of steam-exploded wood

Table 26. Sugar utilization and ethanol production by Z. mobilis and S. cerevisiae grown on acid hydrolyzed steam-exploded Aspen fractions

Table 27. Recovery of glucose and conversion to ethanol by Z. mobilis grown on acid hydrolyzed steam-exploded wood

Table 28. Comparison of the percent conversion of cellulose to ethanol between acid and enzymatic hydrolysis

Table 29. Effect of steam-exploded Aspen samples on the ethanol production of Zymomonas mobilis and Saccharomyces cerevisiae grown on 5% glucose

Table 30. Effect of steam-exploded Aspen samples on the production of ethanol under finite air conditions as compared to a N_2 atmosphere

Table 31. Ethanol production by Z. mobilis and S. cerevisiae in the presence of the water extract from steam-exploded Aspen

List of Figures

Figure 1 Effect of storage on β-glucosidase activity of T. reesei C30

Figure 2 Effect of storage on filter paper activity of T. reesei C30

Figure 3 Effect of storage on endoglucanase activity of T. reesei C30

Figure 4a Thermal stability of endoglucanase activity of the culture filtrate from T. harzianum E58

Figure 4b Thermal stability of endoglucanase activity of the culture filtrate from T. harzianum E58

Figure 5a Thermal stability of β-glucosidase activity of the culture filtrate from T. harzianum E58

Figure 5b Thermal stability of β-glucosidase activity of the culture filtrate from T. harzianum E58

Figure 6a Thermal stability of filter paper activity of the culture filtrate from T. harzianum E58

Figure 6b Thermal stability of filter paper activity of the culture filtrate from T. harzianum E58

Figure 7a Thermal stability of endoglucanase activity of the culture filtrate from T. harzianum E58 after filtrate is adjusted to a pH of 4.8

Figure 7b Thermal stability of endoglucanase activity of the culture filtrate from T. harzianum E58 after filtrate is adjusted to a pH of 4.8

Figure 8a Thermal stability of β-glucosidase activity of the culture filtrate from T. harzianum E58 after filtrate is adjusted to a pH of 4.8

Figure 8b Thermal stability of β-glucosidase activity of the culture filtrate from T. harzianum E58 after filtrate is adjusted to a pH of 4.8

Figure 9a Thermal stability of filter paper activity of the culture filtrate from T. harzianum E58 after filtrate is adjusted to a pH of 4.8

Figure 9b Thermal stability of filter paper activity of the culture filtrate from T. harzianum E58 after filtrate is adjusted to a pH of 4.8

List of Figures Cont'd

Figure 10. Effect of pH on the activities of endoglucanase (top), β-glucosidase (middle) and filter paper (bottom) cellulase components of T. harzianum E58. Two buffer systems were employed; pH 4.2-5.6 was achieved using sodium acetate - acetic acid while pH 5.2-6.8 was achieved using citric acid-sodium phosphate

Figure 11. Effect of increasing solka floc concentrations on the production of endoglucanase(top) β-glucosidase (middle) and filter paper (bottom) activities by T. harzianum E58

Figure 12. Effect of increasing glucose concentrations on the production of endoglucanase (top), β-glucosidase (middle) and filter paper (bottom) activities by T. harzianum grown on 2% solka floc

Figure 13. Effect of increasing furfural concentrations on the ethanol production by S. cerevisiae and Z. mobilis grown on 5% glucose

SUMMARY

Various parameters affecting the overall conversion of lignocellulosics to ethanol were studied. Of prime importance in a lignocellulosics to ethanol process is the pretreatment of the woody biomass. We have found that high levels of hydrolysis (92%) can be achieved with material that was acid soaked prior to steam-explosion and water extracted after steam explosion. This hydrolysis was accomplished through a combined hydrolysis and fermentation technique which produced, under optimum conditions, a broth containing 2.3% ethanol. Optimum conditions for the combined hydrolysis and fermentation process were found to be a culture pH of 4.8, a hydrolysis temperature of 45^{o}C and a fermentation temperature of 37^{o}C. Yeast extract was found to be inhibitory to the action of the cellulases and was only added following the hydrolysis step.

Production of the cellulase enzymes was found to be dependant on the presence of cellulose in the growth media. Under the conditions reported here, the optimum cellulose level for cellulase production was found to be 1 to 2%. In the case of Trichoderma harzianum E58 highest cellulase levels were produced when a commercially available cellulose source, such as solka floc, was used as the substrate. Attempts to induce high levels of enzyme production from this organism on real lignocellulosics substrate were unsuccessful. Trichoderma reesei C30, on the other hand, produced substantially higher levels of cellulase enzymes when a 60 second water extracted steam-exploded aspen sample was used for growth as compared to when solka floc was used. Very low cellulase enzyme production

were achieved by either T. reesei C30 or T. harzianum when these were grown on unextracted steam-exploded material. The unextracted steam-exploded material also contained material that inhibited the growth of the fermentative organism Zymomonas mobilis. The water extraction procedure removed these substances and our results indicated that water extraction is required before the steam exploded material can be used for the production of either cellulase enzymes or ethanol. The nature of the inhibitory substances are unknown. Furan derivatives, such as furfural, are probably not implicated.

INTRODUCTION

Despite the current surplus of oil in the world market today there are significant social, political and economical reasons why most countries would still like to be self-sufficient in liquid fuels. One of the alternatives receiving the most attention is the production of ethanol from lignocellulosics. The potential that lignocellulosic materials offer is enormous, but the realization of that potential awaits the development of processes that will provide useful products at a cost attractive to the producer and acceptable to the environmentalist. Biotechnological processes, being low in energy input requirements and non-polluting, may satisfy both criteria.

The cellulose in lignocellulosics cannot be easily hydrolysed since lignin encrusts the cellulose fibrils in a sheath-like manner and is physically and chemically bonded to the polysaccharide. This fact necessitates a pretreatment method which can depolymerize or solubilize the lignin before cellulose can be readily hydrolysed. In this manuscript, we report on the pretreatment method of steam explosion as it pertains to the conversion of lignocellulosics to ethanol.

Steam explosion is a pretreatment that essentially involves the steaming of lignocellulosic substrates at a variety of different temperatures, pressures and retention times with a sharp reduction in pressure to expel the material from the vessel. Steam explosion includes both physical and chemical pretreatment. The high pressure steaming of the moist lignocellulosic substrates

results in the partial decomposition of some of the hemicellulose components to acids, mainly acetic acid, which catalyze the depolymerization of the hemicellulose and lignin (Saddler and Brownell, 1982).

Following pretreatment, the lignocellulosic substrate must then be hydrolysed to its respective monomeric sugars. This can be accomplished either through acidic or enzymatic hydrolysis. Enzymatic hydrolysis is normally achieved through the use of cellulase enzymes derived from the fungus Trichoderma. The advantage of using Trichoderma cellulases are that the cellulase system is a complete cellulase system which can hydrolyse cellulose to glucose through the synergistic action of three types of enzymes (Mandels, 1982). The cellulases are excreted extracellularly so that high yields of cellulase proteins are readily available in the culture filtrate. One major disadvantage of this cellulase system is that the accumulated glucose causes end-product inhibition on the β-glucosidase enzymes which in turn inhibits the whole hydrolysis process.

This stumbling block has been circumvented by the procedures of simultaneous saccharification and fermentation (Blotkamp et al 1979 and Ghose et al 1982) or of combined hydrolysis and fermentation (Saddler et al 1982b).

To develop an efficient process for the conversion of lignocellulosics to ethanol using enzymatic hydrolysis it is necessary that a) the substrate first be pretreated b) a highly efficient cellulase system is produced cheaply which is active on the pretreated substrates c) the major part of the cellulose is hydrolysed to glucose and

d) this sugar solution is readily fermented to ethanol. Each of these points was studied and will be discussed in this report.

MATERIALS AND METHODS

Microorganisms:

All the fungi used were taken from the Forintek Culture Collection. Trichoderma reesei QM 9414 and T. reesei C30 were originally supplied by ATCC and Rutgers University respectively. A spore inoculum was used to initiate growth in shake flasks containing Vogels medium (Montenecourt and Eveleigh 1977) and solka floc B.W. 300 (Brown & Co. N.H. U.S.A.) as the cellulose substrate. Saccharomyces cerevisiae C495 (NRCC 202001) was grown under standard conditions (Rainbow, 1970). Zymomonas mobilis Z2 (ATCC 29191) was grown in 60 ml serum vials (Miller and Wolin, 1974) using the medium of Rogers et al (1979).

Cellulase preparations:

The cellulolytic fungi were grown for specified times at 28^{o}C on solka floc. Cultures were then filtered through a Reeve Angel glass fibre filter. In some cases the filtrate was further concentrated on a rotary evaporator and these rotary evaporated samples were sometimes further concentrated with 60% ammonium sulphate. The precipitated cellulase enzymes were resuspended in 0.05M citrate buffer pH 4.8.

Celluclast and cellobiase preparations were obtained by courtesy of Novo laboratories, Wilton, CT. U.S.A. and diluted with 0.05M

citrate buffer pH 4.8 to a protein concentration of 10 mg/ml. A mixture of the celluclast preparation (10 mg/ml) and cellobiase preparation (10 mg/ml) was termed NOVO combination.

Combined hydrolysis and fermentation procedure:

One gram of the cellulosic substrate and 0.1 gm of yeast extract were added to a 60 ml serum vial with 5 mls of distilled water. The vials were then gassed with N_2, capped and autoclaved at 15 psi for 20 minutes. Fifteen millilitres of a predetermined cellulase preparation was added aseptically to initiate the experiment and the vials were incubated at 50°C on an orbital shaker (100 rpm) for 24 hours. One millilitre of a 3 day old S. cerevisiae or Z. mobilis culture was aseptically added after this time and the vials reincubated at 37°C for various times.

Fractionation of fungi:

The cellulolytic fungi were grown on 2% solka floc. At specified times 10 ml samples were withdrawn from each flask and spun at 10,000 rpm for 15 minutes. The supernatant fluid was designated as the filtrate. The pellet was washed in citrate buffer pH 4.8 and then resuspended in 10 mls of citrate buffer pH 4.8 and sonicated until the cells lysed. The sample was again centrifuged at 10,000 rpm for 15 minutes and the resulting supernatant fluid designated as cell residue. The pellet was resuspended in 10 mls citrate buffer pH 4.8 and designated as the cell wall.

Assays:

Soluble protein was determined by the method of Lowry et al. (1951) using bovine serum albumin (SIGMA) as the standard. Total reducing sugars were estimated colorimetrically using dinitrosalicylic acid reagent (Miller, 1959). Glucose was determined colorimetrically by the glucostat enzyme assay (Raabo and Terkildsen, 1960).

Filter paper activity was determined by the method of Mandels et al (1976). One millilitre of properly diluted culture supernatant was added to 1 ml of 0.05M citrate buffer pH 4.8 containing a 1 cm x 6 cm strip (50 mg) of Whatman No. 1 filter paper. After incubation for 1 hour at 50°C, the reaction was terminated by the addition of 3 mls of dinitrosalicylic acid reagent. The tubes were placed in a boiling water bath for 15 minutes then cooled to room temperature and the absorbance read at 575 nm. One unit of filter paper activity was defined as 1 µM of glucose equivalents released per minute.

Endoglucanase activity (1, 4.β-D-glucan 4-glucanohydrolase, EC 3.2.1.4) was determined by incubating 1 ml of appropriately diluted culture filtrate with 10 mg of carboxymethyl-cellulose (CMC medium viscosity, Sigma No. C-4888) in 1 ml of 0.05M citrate buffer pH 4.8 at 50°C for 30 minutes. The reaction was terminated by the addition of 3 ml of dinitrosalicylic acid reagent. One unit of endoglucanase activity is defined as 1 µM of glucose equivalents released per minute. β-glucosidase activity (E.C. 3.2.1.21) was determined by incubating 1 ml of appropriately diluted culture filtrate with 10 mg of salicin (Sigma) in 1 ml of 0.05M citrate buffer pH 4.8 at 50°C for 30 minutes. The procedure followed was as for the endoglucanase assay. One unit of β-glucosidase activity is

defined as 1 μM of glucose released per minute.

Ethanol was determined by gas-liquid chromatography using a 6 ft x 1/8 in Chromosorb 101 column with flame ionization detector. The detector was kept at 250°C and the column oven operated at 160°C with the injector port at 170°C. Helium which was saturated with formic acid (Ackman, 1972) was used as the carrier gas at a flow of 30 ml/min.

Pentosans were assayed by the method outlined in TAPPI (Technical Association of the Pulp and Paper Industry) standard T223-05-71, acid-insoluble lignin was assayed by the method outlined in TAPPI standard T222-05-75.

Acid hydrolysis of wood samples was performed essentially as in TAPPI standard T222-05-74 except that a lower ratio of acid to wood was used as in the hydrolysis procedure according to Saeman et al (1954). The resulting sugar syrup was deacified through the use of Amberlite IR-45 resin. The monosaccharides were detected with a Varian 5000 high pressure liquid chromatograph using a Biorad Aminex HPX-87P heavy metal form column with a series of guard columns designed to de-ash the samples. The solvent system was water at a flow rate of 0.6 ml/min and the column was kept at 85°C. Detection of the monosaccharides was accomplished with the use of a refractive index detector maintained at a constant temperature.

Substrates:

Solka floc B.W. 300 F.C. was purchased from Brown and Co., N.H., U.S.A.

Aspen wood samples were steam exploded using a high pressure gun (Saddler et al, 1982b).

a) SE - steam exploded wood chips which had been exposed to saturated steam at 560 psi

b) SEW - Above fraction which was then extracted at room temperature for 2 hours with water

c) SEWA - Samples which had been treated as in section B were further extracted for 1 hour with 0.4% NaOH, washed thoroughly with water, mildly acidified with dilute H_2SO_4 and again washed until the samples were neutral

d) SEWAC - Samples which had been pretreated as in section c) and further treated with 2% of their weight with sodium chlorite

The moisture content of the samples was measured before storing them at -10°C.

RESULTS

1. Location of cellulase enzymes:

The majority of the cellulase enzymes of T. reesei are extracellular and found in the culture filtrate. However, β-glucosidase is known to be localized within the cell (Steinberg and Mandels, 1982) and is not removed by treatments which have been found to release enzymes from the periplasmic spaces of fungi (Lisker et al, 1975). In order to locate the cellulase enzymes, we grew T. reesei C30 on various substrates and fractionated the cells. Results in Table 1 revealed that the majority of the endo- and exo-cellulase enzymes were extracellular and secreted into the culture filtrate. The β-glucosidase enzyme was, however, found within the cell. There appeared to be a substantial amount of filter paper activity associated with the cell residue and this could be due to the high β-glucosidase activity which is known to effect the filter paper assay (Joglekar et al 1983). When T. reesei C30 was grown on cellobiose instead of solka floc, there was still substantial cellulase activity produced.

When the same series of experiments were repeated with T. harzianum E58 (Table 2), the cellulase enzyme production was quite minimal on both cellobiose and glucose. Also, the low levels of cellulase enzymes in the culture filtrate was not due to non-secretion of these enzymes but to a lack of enzyme production. When T. harzianum E58 was grown on solka floc, the majority of the cellulase enzymes were found in the culture filtrate. The β-glucosidase levels in the culture filtrate were substantially increased in this culture

filtrate over those levels found in the culture filtrate of T. reesei C30.

2. Concentration and storage of cellulase enzymes:

In any process involving cellulose hydrolysis based on enzymes, there will be a requirement for an efficient means of concentrating and storing these enzymes. We have tested two methods of concentration, that of rotary evaporation and ammonium-sulphate precipitation for both T. reesei C30 (Table 3) and T. harzianum E58 (Table 4).

For the cellulase filtrate from T. reesei C30, both rotary evaporation and ammonium-sulphate precipitation increased the specific activity of all three cellulase components. The highest increases in specific activity were with the ammonium sulphate precipitated technique, probably since this technique also purifies the enzyme preparation by removing soluble sugars and other extraneous material. For the cellulase filtrate from T. harzianum E58, rotary evaporation proved to be slightly detrimental to the specific activities of the cellulase components. As was the case with T. reesei C30, ammonium-sulphate precipitation also increased the specific activities of the T. harzianum E58 cellulase enzymes.

When we tested the effect of different storage conditions on these concentrated enzyme preparation, we found that the enzymes which had been concentrated by rotary evaporation lost activity when stored in a frozen state but did not lose activity when

stored at 4°C (Table 5). For the cellulase enzymes which had been concentrated by ammonium sulphate precipitation, storage under either of the two conditions increased the specific activities of all the cellulase components. In another experiment, the cellulase enzymes derived from T. reesei C30 were stored under various conditions and in different concentrated forms for a period of 77 days (Figure 1,2,3). The results in Figure 1 showed that β-glucosidase activity increased during storage at 4°C but decreased when stored frozen. The filter paper activity (Figure 2) appeared to remain stable under all storage conditions while the endoglucanase activity (Figure 3) remained stable in all cases except as a rotary evaporated concentrated form stored at 4°C. From this data it appears there is a trend which indicates that freezing is detrimental to β-glucosidase activity and that storage at 4°C over an extended period of time is detrimental to endoglucanase activity when the culture filtrate has been concentrated by rotary evaporation.

Since ammonium-sulphate precipitation seemed to increase the specific activity of our cellulase filtrates we assayed for other methods of purification. Rotary evaporated culture filtrates were precipitated with either ammonium-sulphate, ethanol or acetone. Results in Table 6 revealed that the β-glucosidase was stable under all three modes of purification, however, both endoglucanase and filter paper activity were severely affected by either ethanol or acetone precipitation.

3. Thermal stability of cellulase enzymes

The optimum temperature for the cellulase assays were developed for the cellulase system of T. reesei QM 9414 (Mandels et al, 1976). Since we were working primarily with cellulase enzymes derived from T. harzianum E58, preliminary experiments were carried out to test the optimum temperature for the assay of the endoglucanase, β-glucosidase and filter paper activities. From data presented in Table 7, it was concluded that although the standard assay methods were not at optimum temperatures for cellulases from T. harzianum E58, they were acceptable.

The pH optimum for each assay was also tested and found to be around pH 4.8 (Figure 4). The ionic strength and type of buffer seemed to have a substantial influence on the activity of all three cellulase enzyme components. This fact needs to be remembered when data from various laboratories are compared, especially if non-standard methods are used for assaying enzyme activities.

The thermal stability of the various cellulase components over extended time periods (i.e. 1-3 days) under actual hydrolysis conditions was also tested. The endoglucanase activity of the culture filtrates remained stable at 28°C for up to 3 days but the stability was greatly reduced after incubation at 37°C with only 40% activity remaining after 3 days (Figure 5a). The thermal stability of the endoglucanse at 50° and 60° was very poor such that the endoglucanase activity dropped drastically even after 30 minutes incubation (Figure 5b). The same general trend was apparent for both β-glucosidase activity Figures 6a & 6b) and filter paper activity (Figures 7a & 7b).

These results were of interest as under normal hydrolysis conditions carried out at 50°C, the activity of the cellulase enzymes was almost completely lost after 30 minutes. Since these results conflicted with known results obtained after 1-3 days hydrolysis, the experiment was repeated but this time the pH of the culture filtrate was adjusted to 4.8. T. harzianum E58, unlike T. reesei C30 does not show a rapid drop in pH during growth on solka floc and the pH of the culture filtrate remained at about 6.8. The thermal stability of all three cellulase components was far superior once the pH of the culture filtrate was adjusted to 4.8 (Figures 8a,b, 9a,b, 10a, b). The most susceptible component appeared to be the β-glucosidase activity (Figure 9b) which showed poor stability at 50°C but acceptable stability at 45°C. This instability of β-glucosidase at 50°C was sufficient to warrant a review of our hydrolysis temperature. A 24 and 48 hour hydrolysis was performed at various temperatures ranging from 30°C to 50°C. The results on Table 8 show that following a 24 hour hydrolysis, maximum reducing sugars were achieved at an incubation temperature of 37° while maximum glucose was achieved after incubation at 50°C. After a 48 hour hydrolysis, maximum reducing sugars were achieved at the hydrolysis temperature of either 37° or 45° while maximum glucose was achieved at a temperature of 45°C. In order to ascertain the best hydrolysis temperature in a combined hydrolysis and fermentation procedure, various hydrolysis temperatures between 37° and 50°C were tested followed by fermentation at 37°C (Table 9). Highest ethanol values were achieved following a hydrolysis temperature of either 37°C or 45°C. We also compared final ethanol values

between a simultaneous saccharification and fermentation (i.e. both the cellulolytic enzymes and the fermentative organism are added at the beginning of the procedure) and a combined hydrolysis and fermentation procedure. Highest ethanol values were achieved with a combined hydrolysis and fermentation procedure.

4. Production of cellulases:

The eventual production of cellulase is highly dependant on the proper growth conditions of the fungi. Some of the basic problems in the growth of mycelial cultures are the aspects of aeration and agitation which, if not properly adjusted, can cause layering and subsequent regions of starvation. These two factors are directly related to the ratio between surface to volume and restrict the amount of material which can be handled by a standard conical flask. In order to determine the correct surface to volume ratio necessary for maximal cellulase production by T. harzianum E58, under the conditions described in the Materials and Methods section, we varied the volume of media in various sized flasks and measured the production of cellulases (Table 10). Although the ratio of media volume to flask size remained constant in the first four entries of Table 10, the extracellular protein and the specific cellulase activity decreased with increasing volume of media. Since the flasks are conical, maximal surface area for each flask is attained at the widest part of the flask therefore the liquid media should not exceed this area. The size of the flask is also a factor in agitation since at a given speed of agitation, a smaller flask will have better mixing than a larger flask.

Taking all these factors into account, cellulase enzymes are routinely produced in 500 ml conical flasks containing 150 mls of liquid media.

Cellulase enzymes derived from T. reesei are inducible enzymes which require a cellulose substrate for their production. Since T. harzianum is a newly studied cellulase producer we have studied the production of extracellular cellulase enzymes when the organism is grown on different growth substrates. Results in Table 11 indicated that for a complete spectrum of cellulase enzymes to be produced, cellulose must be present. After 2 days growth on cellobiose or xylose, high β-glucosidase and filter paper activity were measured by our assay technique. The high filter paper activity could be due to the high β-glucosidase activity which can increase the apparent filter paper activity (Joglekar et al 1983) and to the high background sugars which interfere with this assay. The observation that β-glucosidase was produced when xylose or carboxymethyl cellulose were used as substrate needs to be verified and this should be done by measuring released glucose by the glucostat assay.

Since cellulose is the sole inducer of cellulase enzymes, we determined the concentration of cellulose that could produce maximum cellulase enzymes. Results in Figure 11 revealed that for endoglucanase and filter paper activity, maximum activity was achieved at a 1% solka floc concentration while maximum β-glucosidase activity was achieved at 2% solka floc. It has been reported (Mandels and Reese, 1957) that the addition of small amounts of glucose in the growth medium is beneficial to the production of cellulases since glucose

permits a rapid initial growth of the Trichoderma and reduces the lag phase. In order to test this, we included various levels of glucose from 0.01% to 1.0% to our basic media containing 2% solka floc. Our results showed (Figure 12) that the addition of 0.01% or 0.1% glucose was beneficial to the endoglucanase and filter paper activity while slightly detrimental to the β-glucosidase activity. Higher levels of glucose prove to be detrimental to all cellulase enzyme activities.

One of the major costs involved in the production of cellulases is the cost of a pure cellulose such as solka floc. We have carried out extensive investigations aimed at replacing this commercially available substrate with a cheaper substrate such as steam exploded aspen. As a preliminary experiment we grew T. reesei QM 9414, C 30 and T. harzianum E58 on 5% steam exploded wood which had been untreated and steam exploded wood which was water extracted and alkali-treated. The extracellular protein values given in Table 12 are abnormally high when steam-exploded wood was used as the substrate and this was due to the presence of lignin derivatives which interfere with the Lowry protein assay. For this reason enzyme activities from steam-exploded wood are always given as IU/ml. From Table 12 we can see that in general, the steam-exploded wood is not a good substrate for enzyme production even after it is water extracted and alkali treated. Since this experiment was performed with the fairly high substrate concentration of 5% we repeated the experiment using a 2% substrate concentration. The results

from Table 13 also indicate the effect of various extraction procedures on the overall production of cellulases. For all three fungi tested the highest cellulase activity was obtained from the most processed wood sample, the steam-exploded water extracted and alkali treated samples. When Trichoderma D39 was used, there was little difference in cellulase production using either the steam-exploded water extracted samples and the more processed steam-exploded water and alkali extracted fractions. T. reesei C30 and T. harzianum E58 were further studied for their cellulase enzyme production on various fractions of steam-exploded wood as compared to the cellulase enzyme production on a commercial available cellulose such as solka floc. From Table 14 we can see that cellulase production with the organism T. reesei C30 was highest when this organism was grown on 2% solka floc when compared to 2% pretreated wood substrates. The lowest enzyme production was found when 2% steam-exploded wood was used as the substrate. When steam-exploded wood which had been water and alkali extracted was used as the substrate there was a drop of 18% in endoglucanase activity as compared to solka floc, a 43% drop in β-glucosidase and a 39% drop in filter paper activity. When steam-exploded water and alkali extracted wood was used as the substrate, peak enzyme activity was achieved earlier as compared to that found with solka floc. The further pretreatment of chloriting does not enhance the enzyme production over levels achieved prior to chloriting. When the same substrates were used for growth of T. harzianum E58, the results (Table 15) followed the same trend but enzyme production using steam-exploded water extracted and alkali treated wood as the substrate was reduced by 46% in endoglucanase actívity and 60%

in filter paper activity while β-glucosidase activity was only decreased by 16%. The cellulase enzyme production with T. harzianum E58 peaks at earlier times than the production from T. reesei C30 and also falls off very rapidly after peak activities were attained. In the two previous experiments the exploded wood had been steamed for 20 seconds. We then assayed for enzyme production after growth on wood which had undergone a longer steam treatment. Results reported in Table 16 are an assessment of the enzyme production of T. reesei C30 on 2% wood substrate which was steamed for 60 seconds, compared to the amount of enzyme produced on 2% solka floc. All extracted exploded wood fractions proved to be better substrates for enzyme production than solka floc. Highest enzyme production was achieved when steam-exploded water extracted wood was used as a substrate. In this sample, the filter paper activity was increased by 309% over that achieved by solka floc, the β-glucosidase was up by 257% and the endoglucanase was increased by 167%. This remarkable increase in cellulase enzyme production would appear to place lignocellulosics in a favourable light as the substrate of choice for the production of cellulase enzymes. These high enzyme levels were not achieved with T. harzianum E58 (Table 17) when this experiment was repeated using this organism. Although enzyme levels were higher than those found in the 20 second steam treatments they still did not match levels achieved on solka floc.

5. Enzymatic Hydrolysis of Lignocellulosics and Conversion of Sugars to Ethanol:

As a preliminary experiment in the enzymatic hydrolysis of lignocellulosics, we compared the reducing sugars and glucose released from two wood substrates by the hydrolysis of NOVO-combo cellulase enzymes and the cellulase enzymes derived from T. harzianum E58. Both cellulase preparations were used at high enzyme activity levels and the results are presented in Table 18. The NOVO-combo cellulase enzymes achieved higher levels of hydrolysis than did the cellulase enzymes from T. harzianum E58. Previously it was reported (Saddler et al 1982) that optimum cellulose hydrolysis was achieved with steam-exploded wood which had been exposed to 20 seconds of steaming. As a follow-up to this information we wished to determine which extraction method was best for maximal hydrolysis and production of reducing sugars. The four fractions tested were unextracted steam-exploded wood, water extracted steam exploded wood, water extracted and alkali treated steam-exploded wood and water extracted, alkali treated and chlorited steam exploded wood. The chemical composition of these fractions is given in Table 19. Based on this table we can see that water extraction serves mainly to remove soluble pentosans and some acid soluble lignin, subsequent alkali treatment removes most of the Klason lignin fraction while further chloriting removes all the acid soluble lignin and some more Klason lignin. The cellulose content in these fractions is increased with each treatment since these extraction procedures leave cellulose

untouched. Each fraction was enzymatically hydrolyzed by T. harzianum E58 at a 5% cellulose concentration and with 27 IU Filter Paper activity/gr dry weight cellulose. The fractions were hydrolyzed under two hydrolysis conditions. The first condition consisted of those temperatures normally used during a combined hydrolysis and fermentation procedure which were 50°C for 24 hours and then 96 hours at 37°C. The second set of conditions were a hydrolysis temperature of 50°C for 120 hours. From the results presented in Table 20 we can see that higher glucose and reducing sugar values were achieved with hydrolysis at the higher temperature for 120 hours. This table also showed that the % hydrolysis increased with the most extracted steam-exploded wood sample and that solka floc had the best % hydrolysis. This seemed to indicate that the cellulase enzymes from T. harzianum E58 were better suited hydrolysing the purer forms of cellulose. We were concerned with the low level of hydrolysis (52%) of solka floc that was achieved with the cellulase enzymes derived from T. harzianum E58. On examining our hydrolysis procedure and comparing it to other procedures we decided that the addition of yeast extract to the enzyme hydrolysis might be affecting the final hydrolysis.

To test this theory we ran a combined hydrolysis and fermentation experiment in which various levels of yeast extract were added. The results presented in Table 21 clearly show that yeast extract does inhibit hydrolysis. It was also indicated that yeast extract was not required for growth of S. cerevisiae and this implies that enough growth factors exist in the cellulase preparation to support growth of the fermentative yeast.

The success of steam-explosion method as a pretreatment method is based in part on chemical reactions which occur at the high cook temperatures and which are dependant on the acetic acid derived from the acetyl groups which form part of the hemicellulose. This acid is in part responsible for the solubilization of hemicellulose during steam-explosion. We felt that this preliminary mild-hydrolysis could be increased by presoaking the wood chips in sulphuric acid prior to steaming at 20 seconds. Table 22 presents the chemical composition of these acid soaked samples following steam-explosion and subsequent extraction procedures. These results were used to tabulate values found in Table 23 which compare the effectiveness of water extraction and subsequent alkali treatment between various levels of acid used as a presoak. From this we can see acid soaking the wood prior to explosion increased the effectiveness of both the water extraction to remove hemicellulose and the alkali treatment as a means of removing lignin. This effectively results in wood samples which contained over 90% cellulose.

These wood samples were enzymatically hydrolyzed and fermented to ethanol using the combined hydrolysis and fermentation procedure. The results are presented in Table 24. For most samples, the fermentative organism, Z. mobilis, achieved higher ethanol production than S. cerevisiae. The % conversion of cellulose to ethanol was 68% in the case of solka floc. High conversion figures were obtained with either of the water extracted or the water and alkali extracted wood. The steam-exploded material had quite poor conversion of cellulose to ethanol and this is due in part to a

lower hydrolysis rate and the presence of inhibitors which did not permit the complete utilization of the glucose by Z. mobilis. For the water extracted and the subsequent alkali treated wood the combined hydrolysis and fermentation procedure increased the percent conversion of cellulose by 34% over a straight hydrolysis procedure. This indicates the beneficial effect that continual removal of glucose from the reaction mixture has on the efficiency of hydrolysis.

6. Acid Hydrolysis and Fermentation to Ethanol:

For comparison purposes, the steam-exploded wood samples were also subjected to an acid hydrolysis using a modification of the Klason method. The sugars produced in this way were then fermented to alcohol. Results in Table 25 report the recovery of sugars following the acid hydrolysis of the steam-exploded wood and the various extracted samples. These sugar syrups were then used as substrates for ethanol production by Z. mobilis and S. cerevisiae. These results are presented in Table 26. S. cerevisiae gave poor ethanol values which was probably due to a poor inoculum source since the glucose control also gave poor ethanol values. The steam-exploded acid hydrolyzed samples still contained some inhibitory material as both Z. mobilis and S. cerevisiae gave poor ethanol values. Results in Table 27 report the recovery of glucose and potential ethanol by acid hydrolysis. The recovery of glucose by this method

was between 69 and 78%. Highest ethanol production was achieved from the steam-exploded water extracted and alkali treated fraction. Results in Table 28 compare the percent conversion of cellulose to ethanol using either an acid or an enzymatic hydrolysis. The results for both hydrolysis procedures were very similar. The enzymatic hydrolysis was achieved using culture filtrate from T. harzianum E58 while the acid hydrolysis was performed under the best possible conditions and the sugar syrups passed through an ion exchange resin. In a commercial acid hydrolysis far more sugar is destroyed during the process than the acid hydrolysis performed here would indicate.

In both cases the steam-exploded wood contained an inhibitor which persisted throughout the hydrolysis procedure and inhibited the final production of ethanol.

7. Inhibitors:

From previous experiments there was sufficient data to indicate production of an inhibitor during the steam-explosion process. To further investigate this phenomenon, various steam-exploded samples were incubated with 5% glucose and either S. cerevisiae or Z. mobilis. In this way, we could determine which wood samples contained inhibitory substances. From Table 29 we can see that although S. cerevisiae is not inhibited by any of the wood fractions, Z. mobilis is severely inhibited by the straight steam-exploded material. This inhibition is removed during the water extraction and subsequent alkali treatment. This experiment was repeated and this time we compared the effect of finite air to a pure N_2 atmosphere. It was felt that finite air

conditions may increase the growth of the organism so as to overcome the inhibition caused by the substrate. From data in Table 30 it was shown that finite air as opposed to N_2 had no effect on the inhibition caused by steam-exploded wood. This table also shows that the inhibitor was removed by water extraction. This water-extract was rotary evaporated to near dryness and then tested for inhibition towards either Z. mobilis or S. cerevisiae (Table 31). Neither organism was inhibited by this water extract fraction indicating that the inhibitory substance is somewhat volatile.

It has been suggested (Azhar et al 1981) that the inhibitory substances produced during steam-explosion is furfural. This suggestion was studied by testing the inhibition of furfural towards the sugar consumption and the ethanol production of Z. mobilis and S. cerevisiae. From Figure 13 we can see that even at furfural concentrations of 5 mg/ml ethanol production by S. cerevisiae was only reduced by 55% and only by 60% for Z. mobilis. Further experimentation is needed to pinpoint the nature of the inhibitory substances produced during steam-explosion.

DISCUSSION:

A comparison between T. reesei C30 and T. harzianum E58 revealed that T. harzianum E58 produced β-glucosidase extracellularly and thus levels of this enzyme are always higher in culture broths derived from T. harzianum E58 as opposed to culture broths from T. reesei C30. The regulation of cellulase production is also different between the two organisms in that T. reesei C30 produces substantial cellulase activity even when cellobiose or glucose is used as the substrate. T. reesei C30 is a hyperproducer mutant which resulted from a selective screening and mutation program. (Montenecourt and Eveleigh, 1978).

For both T. reesei C30 and T. harzianum E58 cellulases, the concentration method of choice is the method of ammonium sulphate precipitation. The method not only serves to concentrate the cellulase enzymes but also purifies the preparation by removing soluble sugars. Rotary evaporation appeared to be slightly detrimental to specific enzyme activity particularly for the β-glucosidase activity which is the cellulase component that is most sensitive to heat.

We have studied storage of the cellulase enzymes at either $4^{o}C$ or in the frozen state and found that both storage conditions pose problems. Storage under frozen conditions appears to be detrimental to β-glucosidase activity while storage at $4^{o}C$ may adversely affect the endoglucanase activity. The question of storage needs to be further examined in that other techniques such as freeze drying should also be tested. An aspect of the effect of storage of cellulase enzymes filtrates that may prove to be a problem is

bacterial contamination of the filtrate. This contamination of the filtrate may interfere with both the hydrolysis procedure and the subsequent production of ethanol. Long-term storage of the culture filtrates may not be feasible without prior purification of the enzyme system or sterilization of the culture broths.

Our experiments on the thermal stability of the various cellulase enzymes under use conditions clearly demonstrated the need for a pH of 4.8 prior to use. The instability of the culture filtrate at pH values normally found in the culture broth indicates that for highest enzyme activity, the culture must be harvested at peak times since the cellulase levels will drop with prolonged exposure to the incubation temperatures. The analysis of thermal stability also lead us to re-evaluate the hydrolysis temperature used in a combined hydrolysis and fermentation procedure. Our procedure now calls for a hydrolysis temperature of $45^{o}C$ for 24 hours and then a fermentation temperature of $37^{o}C$.

Blotkamp et al (1979) and Ghosh et al (1982) favour the use of a simultaneous saccharification and fermentation procedure while Saddler et al (1982b) favor a combined hydrolysis and fermentation approach. Both methods achieve the same result which is the removal of glucose from the hydrolysis reaction. Under our conditions, highest ethanol values were achieved with a combined hydrolysis and fermentation approach.

Any industrial process which converts lignocellulosics to ethanol using enzymatic hydrolysis will necessitate the production in house, of cellulase enzymes. Blanch and Wilke, have estimated that

over half the cost of the ethanol produced in this way is due to the high cost of the cellulase enzymes. One very basic aspect in the production of cellulase enzymes are the parameters that affect the growth of the Trichoderma. Two of these parameters are agitation and the mass transfer of oxygen. The main aim of agitation is to provide circulation which overcomes layering and the formation of regions of starvation and, to provide turbulence to reduce the diffusion barrier in the immediate vicinity of cells. Mycelial cultures are a problem since the hyphae are larger and clumping together with layering soon takes place (Calam, 1969). Our experiments have shown that under the conditions we use the ideal sized flask has a volume of 500 mls, is conical and contains 150 mls of media. This series of experiments also provided us with valuable information on the effects of aeration and agitation which will be needed during the eventual scale-up of enzyme production in our 30 litre size fermentor. The experiments which selected the optimum cellulose concentration for enzyme production are also related to the same basic parameters of aeration and agitation. With our fixed parameters of flask size and agitation rate, the ideal concentration of cellulose was found to be either 1 or 2%. At higher substrate concentrations much lower enzyme activities were present in the culture filtrate. Since cellulose is insoluble, aeration and agitation are impeded at high substrate concentration and this in effect, has an adverse effect on growth of the Trichoderma. Cellulose is known to adsorb both endo- and exo-glucanase enzymes (Lee et al, 1982) and this phenomenon also lowered the enzyme activity of culture filtrate at high cellulose concentrations. Therefore, the levels of cellulose

which have been found to be optimum under a laboratory set-up may not necessarily be the same levels of substrate which are found to be optimum in an industrial setting. Work on these basic parameters must be performed in a controlled fermentor.

One of the major costs involved in the production of cellulase enzymes is the cost of a pure substrate such as solka floc. Chahal et al (1981) have reported the use of steam-exploded wood as a substrate for cellulase production by T. reesei C30. We found that with a 2% substrate concentration, highest cellulase activities were achieved with the more processed wood sample. Trichoderma D39 could produce as much cellulase on water-extracted steam exploded wood as on the more processed samples. In this respect, this fungus should be more thoroughly studied since it can produce cellulases when a real lignocellulosic substance is used as the substrate.

We achieved extremely high cellulase activities when steam exploded wood which had undergone a longer steaming process was used as the substrate for enzyme production from T. reesei C30. An increase of 300% in filter paper activity by T. reesei C30 was achieved by when water, extracted steam-exploded wood was used as the substrate as opposed to solka floc. These high values were not achieved by T. harzianum E58 and although the levels of cellulase enzymes were higher for the 60 second steam time than for the 20 second steam time, they were still lower than the levels obtained with solka floc. This whole area needs to be thoroughly researched and the major parameters to consider are length of steaming,

extraction procedure, substrate concentration and *Trichoderma* species.

Our preliminary investigation into the cellulose hydrolysis using cellulase enzymes derived from *T. harzianum* E58 were disappointing in that only low levels of hydrolysis (52%) were achieved using a realistic enzyme level. On re-examining our combined hydrolysis and fermentation procedure we discovered that yeast extract inhibited the action of the cellulase enzymes. In fact, our tests indicate that yeast extract was not required for the growth of the fermentative yeast since sufficient growth factors exist in the cellulase culture filtrate to support growth of the fermentative yeast. This fact would decrease the overall cost of a combined hydrolysis and fermentation process since yeast extract is a costly ingredient. Once yeast extract was eliminated from our hydrolysis procedure our levels of hydrolysis using solka floc as the substrate increased to 68%.

Natural cellulose is a crystalline polymer associated in a matrix with lignin and hemicellulose and is highly resistant to enzymatic attack; therefore pretreatment is necessary. One method which we (Saddler *et al* 1982a) and other workers (Jurasek, 1979; Buchholz *et al*, 1980; Puls and Dietrichs, 1980; Su *et al*, 1980; Taylor, 1980) have been using to enhance conversion of wood substrates is high pressure steaming, with or without fast decompression. The steaming process opens up the cellulose fibers, renders the hemicellulose soluble in hot water and appears to

depolymerize the lignin to some extent (Wayman, 1980). When the conditions are optimized, the lignin becomes readily soluble in dilute sodium hydroxide solution from which it can be recovered as an active chemical by acidification. These properties of steam-exploded lignocellulosics allows us to use the mild extraction procedures of water, followed by alkali, to achieve a fairly purified form of cellulose. To aid in the chemical processes involved in steam-explosion we have found that presoaking the wood in dilute H_2SO_4 greatly increased the efficiency of both the water extraction and the alkali treatment. The percent conversion of cellulose to ethanol using the above fractions in a combined hydrolysis and fermentation procedure was 92% for the water extracted material and 86% for the further alkali treated material.

A comparison of enzymatic hydrolysis versus acid hydrolysis revealed that the percent conversion of cellulose to ethanol were comparable for both methods. These results were achieved using, in the case of the enzymatic hydrolysis, the culture filtrate from T. harzianum E58 while the acid hydrolysis was performed under the best possible conditions.

For both the enzymatic and acid hydrolysis of steam exploded wood there appeared to be material present which inhibited ethanol production by Z. mobilis. This inhibitor was removed by water extraction of the wood and was somewhat volatile. Azhar et al (1981) has reported that severe process conditions during dilute acid hydrolysis of cellulose resulted in the formation of chemicals which were toxic to microorganims. These chemicals were primarily compounds

of the furan series: furfural, 5-hydroxymethylfurfural and 2-hydroxymethylfuran. We tested increasing levels of furfural beyond the level found in our steam exploded samples and found that ethanol production by Z. mobilis was inhibited by 60%. Since the inhibitory material was removed by water extraction, we hope to study this water extract through different fractionation experiments to try and identify the nature of this material. There appears to be a substantial amount of evidence which indicates that it will be necessary to water extract the steam exploded wood before it can be useful in a commercial lignocellulosics to ethanol plant. Our work and work by Chahal et al (1981) indicate that steam exploded wood contains inhibitors not only to the fermentative step of the process but also towards the enzyme production by Trichoderma. Our results also show that with steam-exploded wood we can only achieve low levels of hydrolysis. This fact is substantiated by the work of Sinitsyn et al (1982) which stated that steam-exploded wood contained impurities which inhibit the action of cellulase enzymes. All of this work points towards the necessity of water extraction as a means of removing inhibitors following the steam-explosion process.

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Table 1. Location of cellulase enzymes of Trichoderma reesei C30 grown on various substrates after 8 days incubation

Substrate 2% conc[n]	Fraction[1]	Protein mg/ml	Specific Activity IU/mg		
			Endoglucanase	β-glucosidase	Filter Paper
Solka floc	Filtrate	3.30	15.4	0.20	1.7
	Cell residue	0.10	1.0	0.50	1.0
	Cell wall	0.60	0.6	0.30	0.1
Cellobiose	Filtrate	1.00	24.7	0.1	1.4
	Cell residue	0.40	2.0	0.3	0.4
	Cell wall	2.20	0.1	0.1	-
Glucose	Filtrate	1.0	9.9	0.1	0.6
	Cell residue	0.3	1.7	0.4	0.3
	Cell wall	1.2	0.1	0.1	-

[1] The various fractions were derived as described in Materials and Methods

Table 2. Location of cellulase enzymes of Trichoderma harzianum E58 grown on various substrates

Substrate 2% conc"	Fraction[1]	Incubation Time (days)	Protein mg/ml	Specific Activities IU/mg		
				Endoglucanase	β-glucosidase	Filter Paper
Solka floc	Filtrate	3	0.74	19.5	0.70	0.90
		5	1.33	33.1	1.80	2.15
	Cell residue	3	0.19	1.8	0.60	0.40
		5	0.28	5.9	0.83	0.78
	Cell wall	3	0.13	0.3	0.01	-
		5	1.09	0.9	0.07	0.34
Cellobiose	Filtrate	3	0.57	5.7	0.80	0.60
		5	0.64	3.2	0.10	0.30
	Cell residue	3	0.62	0.2	0.10	0.10
		5	0.50	0.5	0.10	0.30
	Cell wall	3	-	-	-	-
		5	1.33	0.1	0.40	-
Glucose	Filtrate	3	0.60	3.6	0.40	0.30
		5	0.60	2.7	0.10	0.30
	Cell residue	3	-	-	-	-
		5	0.30	0.5	0.50	0.10
	Cell wall	3	-	-	-	-
		5	1.20	0.1	0.10	-

[1] The various fractions were derived as described in Materials and Methods

Table 3. Effect of two concentration techniques on the cellulase enzymes of *Trichoderma reesei* C30

Concentration Technique	Protein mg/ml	Specific activities IU/mg		
		Endoglucanase	β-glucosidase	Filter Paper
Filtrate	1.07	44.1	0.14	1.27
Rotary evaporation	8.04	56.4	0.18	1.73
Ammonium ppt	11.50	69.2	0.20	2.66

Table 4. Effect of two concentration techniques on the cellulase enzymes of Trichoderma harzianum E58

Concentration Techniques	Protein mg/ml	Specific activity IU/mg		
		Endoglucanase	β-glucosidase	Filter Paper
Filtrate	0.83	16.93	0.84	0.66
Rotary evaporation	6.73	13.14	0.74	0.49
Ammonium ppt	5.99	39.61	2.18	1.52

Table 5. Storage of concentrated cellulase enzymes from *Trichoderma harzianum* E58

Concentration Technique	Storage Conditions	Protein mg/ml	Specific activities IU/mg		
			Endoglucanase	β-glucosidase	Filter Paper
Rotary evaporation	Original	6.73	13.14	0.74	0.49
	14 days at 4°C	5.34	17.83	1.05	0.53
	14 days frozen	5.02	16.04	0.41	0.62
Ammonium ppt	Original	5.99	39.61	2.18	1.52
	14 days at 4°C	5.38	50.71	3.50	1.86
	14 days frozen	5.23	49.34	3.52	1.76

Table 6. Purification of concentrated cellulase enzymes from Trichoderma harzianum E58

Purification Method	Protein mg/ml	Specific activity IU/mg		
		Endoglucanase	β-glucosidase	Filter Paper
None	11.0	10.5	0.42	1.02
Ammonium ppt	6.0	29.0	1.39	1.60
Ethanol	6.2	7.8	1.23	0.51
Acetone	7.0	9.0	1.40	0.85

Filtrate was concentrated using rotary evaporation

Table 7. Temperature profile for enzymatic cellulase assays for *Trichoderma harzianum* E58

Incubation Temperature °C	Endoglucanase Activity IU/mg	ß-glucosidase Activity IU/mg	Filter Paper Activity IU/mg
37	10.0	0.37	0.53
45	21.9	0.72	1.04
50	23.4	0.84	1.02
60	21.1	1.50	1.25
70	1.5	-	0.09

Protein concentration: 1.20 mg/ml

Table 8. Production of reducing sugars and glucose from 5% solka floc under various temperatures of hydrolysis

Temperature of Incubation °C	Reducing Sugars mg/ml		Glucose mg/ml	
	Day 1	Day 2	Day 1	Day 2
30	8.3	20.3	3.6	12.8
37	15.4	27.4	9.0	20.8
45	14.1	27.1	10.3	22.5
50	14.8	21.3	11.7	19.2

Cellulase enzymes were derived from culture filtrates of T. harzianum E58

Table 9. Production of ethanol under various hydrolysis temperatures in a combined hydrolysis and fermentation procedure

Hydrolysis Temperature ^{o}C	Reducing sugars[1] mg/ml	Glucose[1] mg/ml	Ethanol[2] mg/ml
37	21.3	10.4	14.6
37°SSF[3]	3.8	tr	13.3
45	22.6	17.2	14.6
50	20.4	16.0	12.3

[1] Reducing sugars and glucose measured after 24 hours hydrolysis
[2] Ethanol produced by S. cerevisiae after 72 hours incubation
[3] SSF - simultaneous saccharification and fermentation, in this case the enzyme and the fermentative organism are added at the same time

Table 10. Production of cellulase enzymes under various media volumes using 2% solka floc

Media Volume (cc)	Flask size (cc)	Protein mg/ml	Specific activities IU/mg		
			Endoglucanase	β-glucosidase	Filter Pape
100	250	1.21	22.6	1.07	1.12
200	500	1.21	25.1	1.20	1.21
400	1000	0.79	11.3	0.87	0.63
800	2000	0.53	3.6	0.33	0.29
1000	2000	0.83	14.7	0.27	0.59
3000	5000[1]	0.82	19.1	0.92	0.81

[1] 5 liter fermentor

Table 11. Production of cellulase enzymes by *Trichoderma harzianum* E58 on different growth substrates

Substrates 2% conc^{n}	Incubation Time (days)	Specific activities IU/mg		
		Endoglucanase	β-glucosidase	Filter Paper
Solka floc	7	33.10	1.64	1.46
Glucose	7	1.80	0.14	0.16
Cellobiose	2	7.48	2.70	1.70
Xylose	2	5.38	2.41	1.35
Xylan	4	2.10	0.26	0.20
CMC	4	5.20	1.88	0.84

Table 12. Production of cellulase enzymes from three different fungi using 5% steam exploded wood as the substrate

Fungi	Substrate	Protein mg/ml	Endoglucanase activity IU/ml	β-glucosidase activity IU/ml	Filter Paper activity IU/ml
Trichoderma reesei QM9414	Solka floc	1.40	62.2	2.10	1.85
	SE[1]	4.90	2.7	0.90	0.70
	SEWA[2]	1.52	23.1	1.20	0.96
Trichoderma reesei C30	Solka floc	2.84	207.4	0.85	5.56
	SE	6.45	3.2	2.96	1.57
	SEWA	2.17	59.3	0.61	2.10
Trichoderma harzianum E58	Solka floc	1.24	52.6	1.96	1.93
	SE	5.03	10.8	1.23	0.62
	SEWA	0.84	2.8	0.55	0.51

The steam-exploded aspen was subjected to 20 seconds steaming at 540 psi prior to explosion. The fungi were incubated for 11 days at 28°C with shaking.

[1] SE - Steam-exploded wood c.f. Materials and Methods
[2] SEWA - Steam exploded wood water extracted and alkali treated c.f. Materials and Methods

Table 13. Production of cellulase enzymes from three different fungi using 2% steam-exploded wood as the substrate

Fungi	Substrate	Protein mg/ml	Endoglucanase Activity IU/ml	β-glucosidase Activity IU/ml	Filter Paper Activity IU/ml
Trichoderma harzianum E58[1]	SE[4]	2.90	11.0	0.44	0.51
	SEW[4]	1.60	21.2	0.89	1.09
	SEWA[4]	1.20	28.0	1.40	1.19
Trichoderma D39[2]	SE	2.20	21.0	0.36	0.93
	SEW	1.46	40.8	1.34	1.71
	SEWA	1.61	41.3	1.45	1.60
Trichoderma reesei C30[3]	SE	4.60	25.8	0.10	1.27
	SEW	3.23	37.7	0.15	1.65
	SEWA	2.90	40.9	0.15	2.51

The steam-exploded aspen was subjected to 20 seconds steaming at 540 psi prior to explosion. The substrate was used at a 2% dry weight concentration and incubated at 28°C with shaking.

[1] Incubation period 6 days
[2] Incubation period 4 days
[3] Incubation period 9 days
[4] SE; SEW; SEWA - steam-exploded wood; steam-exploded water-extracted wood; steam exploded water extracted, alkali treated wood c.f. Materials and Methods.

Table 14. Production of cellulase enzymes from Trichoderma reesei C30 grown on 2% pretreated wood substrate which had been exposed to 20 seconds of steaming

Substrate	Incubation Time (days)	Protein mg/ml	Enzyme activities IU/ml		
			Endoglucanase	β-glucosidase	Filter Paper
Solka floc	7	2.46	164.5	0.30	4.62
	8	2.56	151.0	0.60	5.94
SE[1]	7	3.03	23.4	0.21	0.69
	8	4.36	29.0	0.42	1.38
SEWA[1]	7	2.27	134.7	0.34	3.65
	8	2.03	102.9	0.55	3.26
SEWAC[1]	7	1.80	118.1	0.60	2.36
	8	2.37	108.2	0.45	3.41

[1] SE, SEWA, SEWAC - steam-exploded wood; steam-exploded water extracted and alkali treated wood; steam-exploded water extracted alkali treated and chlorited wood. c.f. Materials and Methods

Table 15. Production of cellulase enzymes from Trichoderma harzianum E58 grown on 2% pretreated wood substrates which had been exposed to 20 seconds of steaming

Substrate	Incubation Time (days)	Protein mg/ml	Enzyme activities IU/ml		
			Endoglucanase	β-glucosidase	Filter Paper
Solka floc	5	1.39	45.0	1.91	2.92
	7	1.35	42.1	2.03	2.29
SE[1]	5	3.38	10.6	0.84	0.82
	7	3.58	14.3	0.90	0.80
SEWA[1]	5	0.92	24.3	1.61	1.16
	7	0.76	4.9	0.23	0.36
SEWAC[1]	5	1.16	20.7	1.18	0.94
	7	1.10	6.4	0.29	0.42

[1]- SE; SEWA; SEWAC - steam-exploded wood; steam-exploded water extracted alkali treated wood; steam-exploded water extracted, alkali treated and chlorited wood c.f. Materials and Methods

Table 16. Production of cellulase enzymes from Trichoderma reesei C30 grown on 2% pretreated wood substrates which had been exposed to 60 seconds of steaming

Substrate	Incubation Time (days)	Protein mg/ml	Enzyme activities IU/ml		
			Endoglucanase	β-glucosidase	Filter Paper
Solka floc	5	1.33	44.4	0.11	1.38
	6	1.48	72.5	0.14	3.08
SEW[1]	5	3.48	64.2	0.37	3.89
	6	3.70	120.8	0.36	9.53
SEWA[1]	5	1.70	50.6	0.24	2.47
	6	1.92	105.0	0.32	6.40
SEWAC[1]	5	1.31	28.9	0.98	1.88
	6	1.86	88.6	0.46	4.54

Wood samples were subjected to 60 seconds of steaming at 560 psi prior to explosion.

[1] SEW; SEWA; SEWAC - steam-exploded water extracted wood; steam-exploded water extracted and alkali treated wood; steam-exploded water extracted alkali treated and chlorited wood. c.f. Materials and Methods

Table 17. Production of cellulase enzymes from *Trichoderma harzianum* E58 grown on 2% pretreated wood substrates which had been exposed to 60 seconds of steaming

Substrate	Incubation Time (days)	Protein mg/ml	Enzyme activities IU/ml		
			Endoglucanase	β-glucosidase	Filter Paper
Solka floc	5	1.28	37.5	1.38	1.88
	6	1.54	44.9	2.76	4.06
SEW	5	2.84	34.2	1.19	1.39
	6	2.45	34.2	1.91	2.87
SEWA	5	1.09	30.3	1.40	1.13
	6	1.12	42.3	2.23	1.75
SEWAC	5	1.11	33.4	1.30	1.21
	6	1.22	53.9	2.14	2.64

Wood samples were subjected to 60 seconds of steaming at 560 psi prior to explosion.

[1] SEW; SEWA; SEWAC - steam-exploded water-extracted wood; steam exploded water extracted and alkali treated wood; steam-exploded water extracted, alkali treated and chlorite wood c.f. Materials and Methods.

Table 18. Production of reducing sugars and glucoses by cellulase enzymes from NOVO and T. harzianum E58

Cellulase source	Substrate 5%	Reducing sugars mg/ml			Glucose mg/ml		
		24 hrs	96 hrs	168 hrs	24 hrs	96 hrs	168 hrs
NOVO	Solka floc	19.5	29.8	44.6	16.3	27.2	38.4
	SE 60 sec[1]	21.8	30.7	33.8	19.0	26.1	24.6
	SE WA 120 sec[2]	14.7	22.9	26.4	12.8	20.8	20.1
E58	Solka floc	8.7	14.2	19.4	6.7	12.3	15.4
	SE 60 sec[1]	17.3	22.5	24.4	10.7	17.6	19.2
	SE WA 120 sec[2]	6.7	8.9	6.4	4.6	7.3	4.8

NOVO activity = 216 FP/gr dry wt
T. harziznum E58 activity = 113 FP/gr dry wt (enzyme profile endoglucanase 19.3; β-glucosidase 0.76; filter paper 1)

[1] - SE-60 sec - Steam-exploded wood exposed to 60 seconds of steaming
[2] - SE WA 120 sec - Steam-exploded water extracted and alkali treated wood which had been exposed to 120 seconds of steaming

Table 19. Composition of steam-exploded Aspen exposed to 20 seconds steaming and following various extractions

Sample	Solid Content % wet basis	Ash %	Pentosan %	Lignin % Klason	Lignin % Acid Sol.	Cellulose %
SE[1]	41.22	0.2	19.32	22.62	2.70	55.16
SEW[1]	23.73	0.2	5.29	24.27	1.90	68.30
SEWA[1]	24.72	0.2	3.74	12.65	1.22	82.19
SEWAC[1]	23.41	0.43	3.57	9.27	-	86.73

[1]- SE; SEW; SEWA; SEWAC: Steam-exploded wood; steam-exploded water extracted wood; steam-exploded water extracted and alkali treated wood; steam-exploded water extracted, alkali treated and chlorited wood c.f. Materials and Methods

Table 20. Production of reducing sugars and glucose by T. harzianum E58 from various pretreated wood substrates

Substrate 5% cellulose	Reducing sugars mg/ml				Glucose mg/ml			
	24 [1]hrs	72 [1]hrs	120 [1]hrs	120 [2]hrs	24 [1]hrs	72 [1]hrs	120 [1]hrs	120 [2]hrs
SE [3]	10.4	9.8	10.1	10.3	1.9	2.9	3.2	3.5
SEW [3]	10.0	10.2	11.8	13.6	6.1	7.6	9.6	11.4
SEWA [3]	8.4	10.6	11.5	14.5	6.6	7.4	10.1	13.1
SEWAC [3]	12.4	15.2	17.0	19.8	11.0	11.5	15.5	18.9
Solka floc	15.9	18.5	23.6	26.3	12.7	13.6	18.5	23.2

27 IU FP/gr dry weight, cellulase profile Endo 27.0: β-glu-0.66; FP. 1.0

[1] First 24 hours hydrolysis was performed at 50°C followed by incubation at 37°C
[2] Hydrolysis performed for full 120 hours at 50°C
[3] SE; SEW; SEWA; SEWAC - steam-exploded wood; steam-exploded and water extracted wood; steam-exploded water extracted and alkali treated wood; steam-exploded water extracted, alkali-treated and chlorited wood, c.f. Materials and Methods

Table 21. Effect of yeast extract on the hydrolysis of 5% solka floc and the production of ethanol following combined hydrolysis and fermentation

Yeast Extract %	Hydrolysis products mg/ml		CHF product mg/ml	% conversion of Cellulose to Ethanol[1]
	Reducing sugar	Glucose	Ethanol	
0	27.2	22.2	17.3	71.1
0.2	24.6	17.9	14.2	58.3
0.5	22.2	14.7	13.9	57.1
1.0	19.1	8.8	14.8	60.8

Combined hydrolysis and fermentation was accomplished using cellulase enzymes derived from T. harzianum E58 and the fermentative microorganisms S. cerevisiae. Hydrolysis was performed at 45°C for 24 hours and then fermentation was allowed to proceed at 37°C for 96 hours.

[1] - % Conversion of cellulose to ethanol = $\frac{\text{Ethanol produced(moles)} \div 1.8 \times 180 \times \frac{162}{180}}{\text{cellulose content}}$

Table 22. Composition of steam-exploded Aspen which was acid-soaked with H_2SO_4 prior to steaming

Sample	Solid Content % wet basis	Ash %	Pentosan %	Lignin % Klason	Lignin % Acid Sol.	Cellulose %
0.2% SE [2]	73.81	-	17.78	23.62	3.43	55.17
0.2% SEW	71.16	-	0.75	31.72	1.96	65.57
0.2% SEWA	75.24	0.26	0.62	7.82	0.44	91.12
0.4% SE [2]	70.61	-	15.37	21.93	7.41	55.29
0.4% SEW	73.33	0.03	0.48	31.07	2.53	65.92
0.4% SEWA	75.04	0.23	0.49	5.52	0.29	93.70

0.2% SE, SEW, SEWA - wood soaked in 0.2% H_2SO_4 then steam exploded (SE); steam-exploded water extracted (SEW); steam exploded water extracted and alkali treated (SEWA) c.f. Materials and Method

0.4% SE, SEW, SEWA - wood soaked in 0.4% H_2SO prior to steam explosion; other symbols (SE, SEW, SEWA) as above

Table 23. Comparison between effectiveness of extraction procedure with and without acid-soaking prior to steam-explosion

Sample	% loss of pentosan	% loss of lignin		% gain cellulose
		Klason	Acid Sol.	
No acid water wash	72.6	+ 7.3[1]	29.6	23.8
alkali	80.6	44.1	54.8	49.0
0.2% acid water wash	95.7	+33.9[1]	42.9	18.5
alkali	96.5	66.9	87.2	65.2
0.4% acid water wash	96.9	+41.7[1]	65.8	19.2
alkali	96.8	74.8	96.1	69.5

[1] gain in Klason lignin content

Table 24. Combined hydrolysis and fermentation of wood which was acid soaked prior to steam explosion at 20 seconds of steaming

Substrate	Fermentative Organism	Reducing Sugar mg/ml	Glucose mg/ml	Ethanol mg/ml	% Conversion Cellulose to Ethanol[1]
02 SE[2]	Control	24.5	4.1	-	-
	SC	18.6	0.2	3.6	25.3
	ZM	18.6	2.2	5.3	37.2
0.2 SEW	Control	24.9	21.5	-	-
	SC	0.9	tr[3]	15.1	89.2
	ZM	1.2	tr	16.7	98.7
0.2 SEWA	Control	25.2	23.2	-	-
	SC	0.7	tr	19.8	84.2
	ZM	1.4	0.2	20.1	85.5
0.4 SE	Control	24.1	6.1	-	-
	SC	22.5	1.1	1.2	8.4
	ZM	20.7	5.0	2.9	20.3
0.4 SEW	Control	25.7	23.5	-	-
	SC	0.9	tr	15.6	91.7
	ZM	1.1	tr	14.7	86.4
0.4 SEWA	Control	27.8	26.1	-	-
	SC	0.7	tr	18.8	77.7
	ZM	1.5	tr	23.2	95.9
Solka floc	Control	35.2	30.0	-	-
	SC	3.5	tr	16.1	62.4
	ZM	6.1	0.8	19.0	73.6

Hydrolysis was achieved at 45°C using cellulase enzymes from T. harzianum E58 at an activity of 40 IU Filter paper/gr dry weight. The cellulase enzyme profile was endo 15.1: β-glu 0.5; Filter paper 1. Fermentation was accomplished at 37° using either S. cerevisiae (SC) or Z. mobilis (ZM). Control samples contained no fermentative microorganism.

[1] % Conversion of cellulose to ethanol: $\frac{\text{Ethanol produced(moles)} \div 2.0 \times 180 \times \frac{162}{180}}{\text{cellulose content}}$

[2] Symbols (SE, SEW, SEWA) same as Table 22

[3] tr - trace

Table 25. Recovery of sugars following acid hydrolysis of steam-exploded wood

Substrate	Starting Weight gr	Sugars recovered g		
		Cellobiose	Glucose	Xylose
SE	10.24	0.34	4.71	2.20
SEW	10.23	0.33	5.32	0.46
SEWA	7.02	0.32	4.95	0.14
SEWAC	6.48	0.24	4.50	0.15

[1] SE; SEW; SEWA; SEWAC: Steam exploded wood; steam exploded and water extracted wood; steam-exploded, water extracted and alkali treated wood; steam-exploded water extracted, alkali treated and chlorited wood c.f. Materials and Methods

Table 26. Sugar utilization and ethanol production by *Z. mobilis* and *S. cerevisiae* grown on acid hydrolyzed steam-exploded Aspen fractions

Substrate	Microorganism	Sugar used mg/ml			Ethanol produced mg/ml
		Cellobiose	Glucose	Xylose	
SE [1]	*Z. mobilis*	2.0	34.5	12.2	11.7
SEW		2.0	51.2	2.6	30.4
SEWA		2.0	49.5	0.4	27.5
SEWAC		1.0	42.0	0.4	22.2
Glucose Control 5%		-	50.0	-	24.6
SE	*S. cerevisiae*	1.7	33.3	11.6	9.2
SEW		1.4	30.8	2.4	11.5
SEWA		1.5	33.8	-	12.9
SEWAC		1.0	42.0	-	11.3
Glucose Control 5%		-	50.0	-	12.4

[1] SE; SEW; SEWA; SEWAC; steam exploded wood; steam-exploded and water extracted wood; steam exploded, water extracted and alkali treated wood; steam-exploded water extracted alkali treated and chlorited wood c.f. Materials and Methods

Table 27. Recovery of glucose and conversion to ethanol by Z. mobilis grown on acid hydrolyzed steam-exploded wood

Substrate	% Recovery glucose (from dry wt)	% Recovery ethanol (based on potential glucose)	g Ethanol/ g cellulose
SE [1]	75.8	37.5	0.21
SEW	69.2	76.4	0.43
SEWA	78.0	83.8	0.48
SEWAC	72.8	68.8	0.39

[1] SE; SEW; SEWA; SEWAC; steam exploded wood; steam-exploded and water extracted wood; steam exploded water extracted and alkali treated wood; steam exploded, water extracted, alkali treated and chlorited wood. c.f. Materials and Methods

Table 28. Comparison of the percent conversion of cellulose to ethanol between acid and enzymatic hydrolysis

Extraction Procedure	% Conversion cellulose to ethanol Acid	Enzymatic
NONE	41.0	37.2
water extraction	86.0	98.7
water extraction followed by alkali treatment	94.2	85.5

Calculation based on results achieved with Z. mobilis as the fermentative organism. The amount of ethanol produced was used to calculate the glucose produced using a conversion of 1.8 moles ethanol/mole glucose.

Table 29. Effect of steam-exploded Aspen samples on the ethanol production of *Zymomonas mobilis* and *Saccharomyces cerevisiae* grown on 5% glucose

Aspen Sample	Microorganism	Glucose used mg/ml	Ethanol produced mg/ml Day 1	Ethanol produced mg/ml Day 3
SE[1]	*Z. mobilis*	ND	0.2	ND
SEWA		40.7	18.9	18.9
SEWAC		38.1	19.2	19.0
NONE		49.1	25.9	23.1
SE	*S. cerevisiae*	40.4	21.5	20.7
SEWA		41.4	21.1	19.3
SEWAC		37.7	19.4	18.7
NONE		47.8	22.1	22.2

[1] SE; SEWA; SEWAC; steam exploded wood; steam exploded water extracted and alkali-treated wood; steam exploded, water extracted, alkali treated and chlorited wood. c.f. Materials and Methods

ND - not detected

Table 30. Effect of steam-exploded Aspen samples on the production of ethanol under finite air conditions as compared to a N_2 atmosphere

Aspen Sample	Incubation Time (days)	% ethanol produced using finite air as compared to N Z. mobilis	 S. cerevisiae
NONE	2	92.3	30.9
	4	94.8	96.3
SE [1]	2	ND	100.0
	4	ND	100.0
SEW	2	81.5	97.9
	4	92.1	106.6
SEWA	2	100.0	99.0
	4	93.8	99.5
SEWAC	2	101.6	107.3
	4	93.8	98.4

[1] SE; SEW; SEWA; SEWAC: steam exploded wood; steam exploded and water extracted wood; steam-exploded, water extracted and alkali treated wood; steam exploded, water extracted, alkali treated and chlorited wood. c.f. Materials and Methods

Table 31. Ethanol production by *Z. mobilis* and *S. cerevisiae* in the presence of the water extract from steam-exploded Aspen

Substrate	Glucose used mg/ml		Ethanol produced mg/ml	
	Z. mobilis	*S. cerevisiae*	*Z. mobilis*	*S. cerevisiae*
5% glucose	49.9	50.0	19.5	20.7
5% glucose & water extract	47.3	47.6	22.3	24.2

Figure 1 - Effect of storage on β-glucosidase activity of T. reesei C30
A - culture filtrate stored at 4°C
B - culture filtrate stored frozen
C - rotary evaporated concentrate stored at 4°C
D - ammonium-sulphate precipitated concentrate stored at 4°C

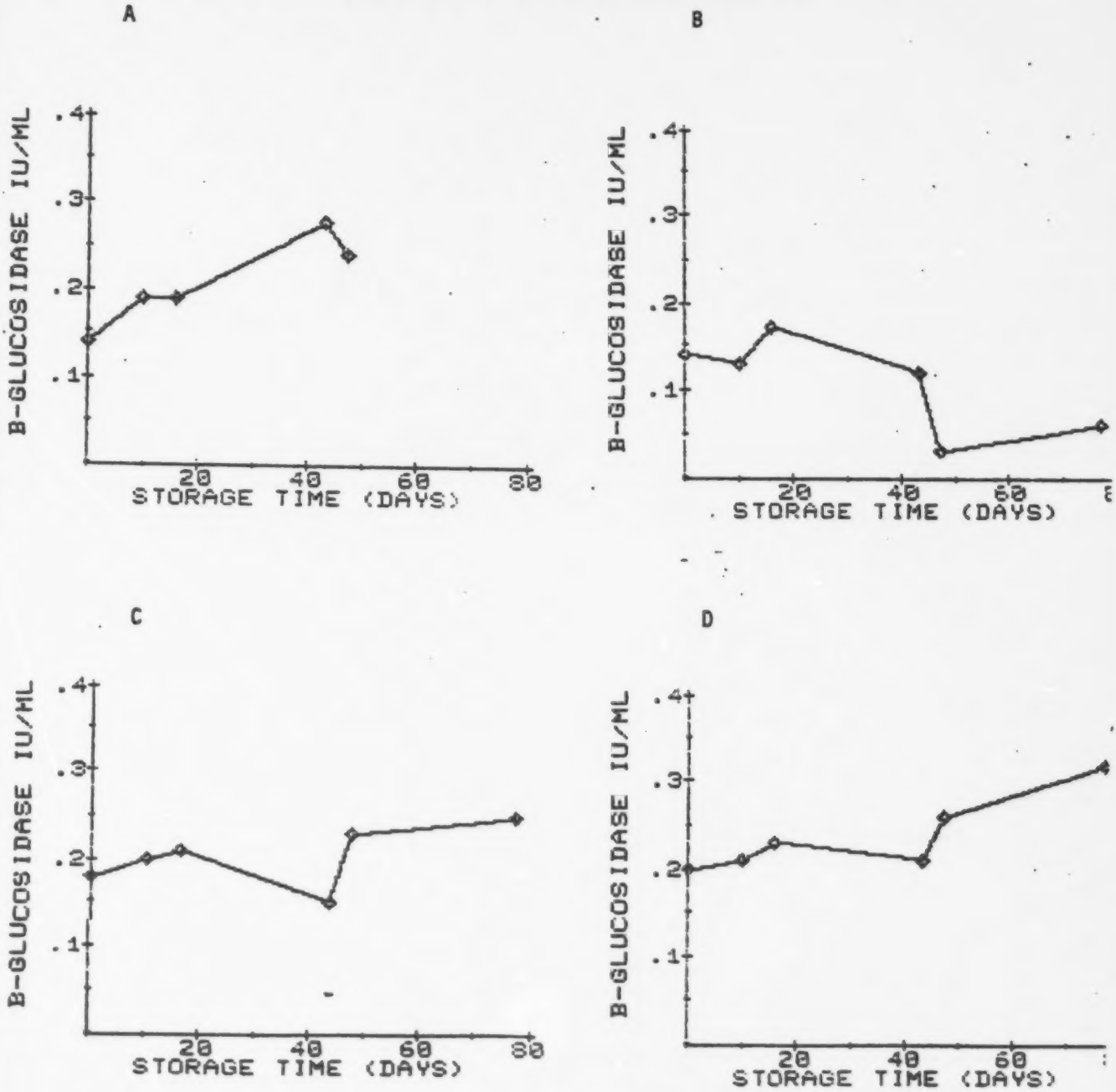
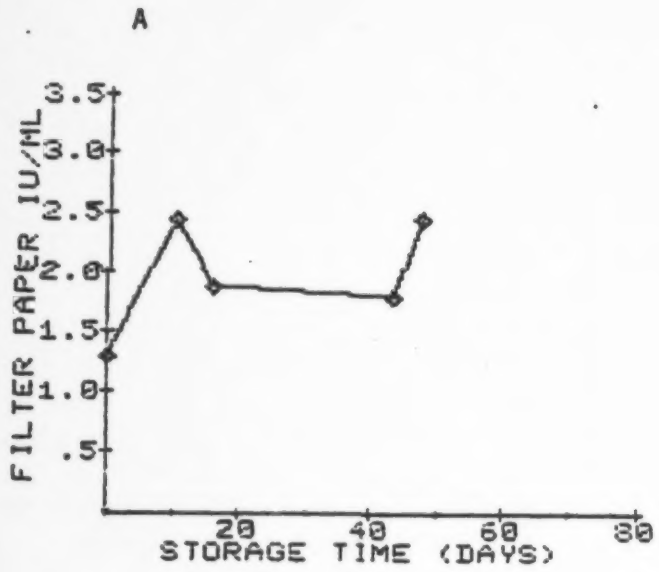
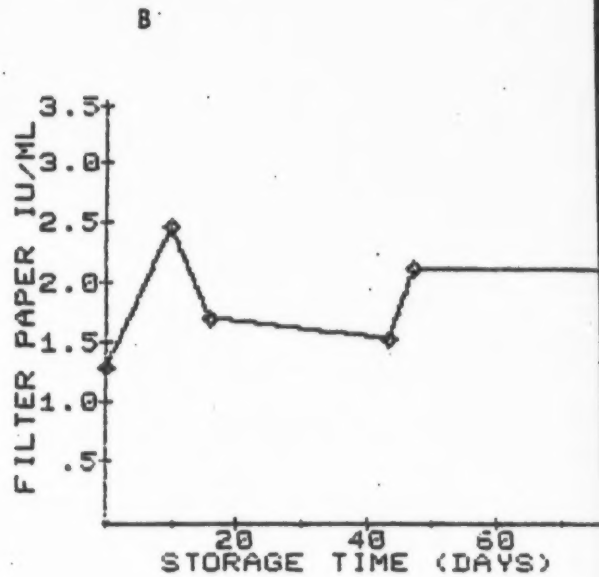
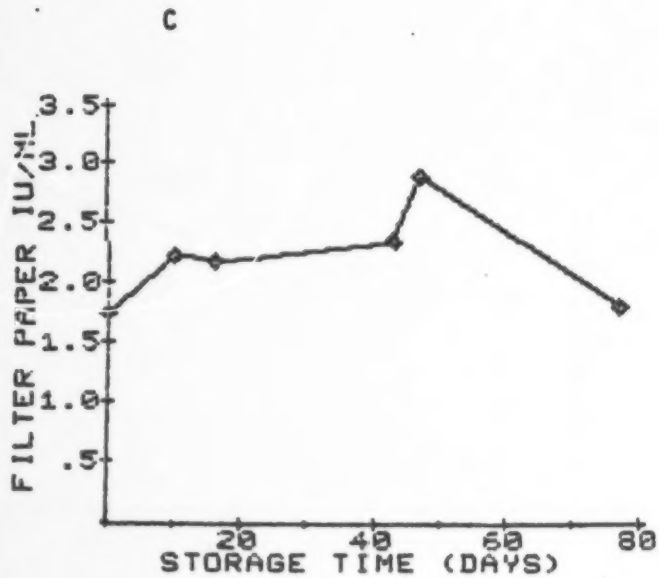


Figure 2. Effect of storage on filter paper activity of T. reesei C30

A - culture filtrate stored at 4°C
B - culture filtrate stored frozen
C - rotary evaporated concentrate stored at 4°C
D - ammonium-sulphate precipitated concentrate stored at 4°C







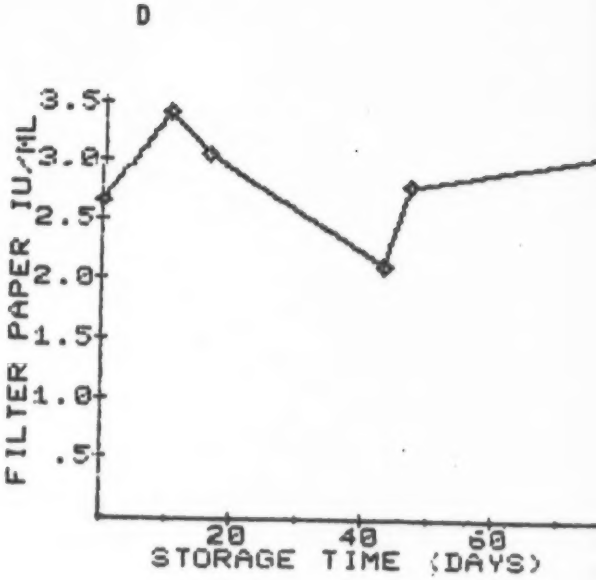


Figure 3. Effect of storage on endoglucanase activity of T. reesei C30
A - culture filtrate stored at 4°C
B - culture filtrate stored frozen
C - rotary evaporated concentrate stored at 4°C
D - ammonium-sulphate precipitated concentrate stored at 4°C

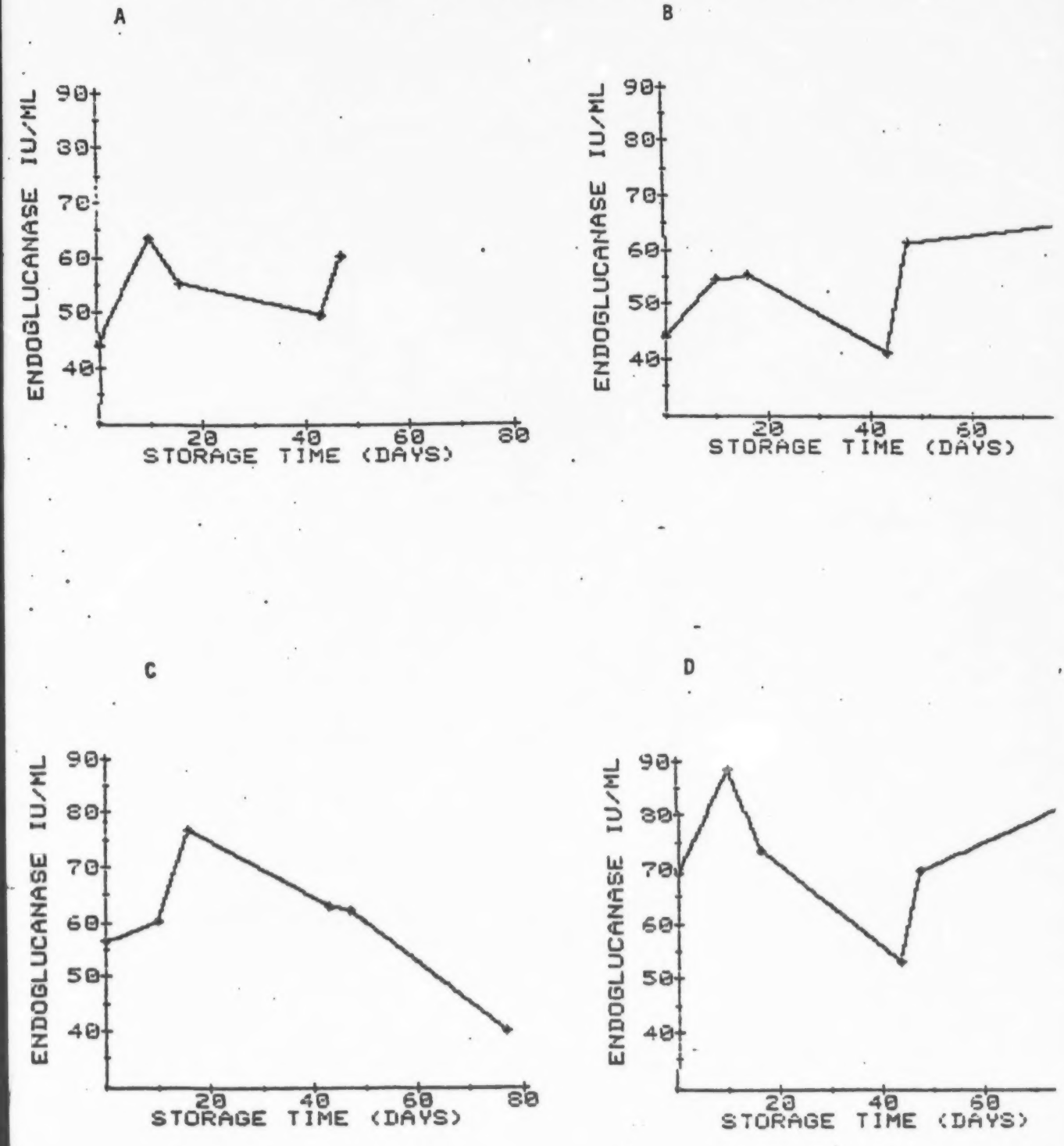


Figure 4a. Thermal stability of endoglucanase activity of the culture filtrate from T. harzianum E58

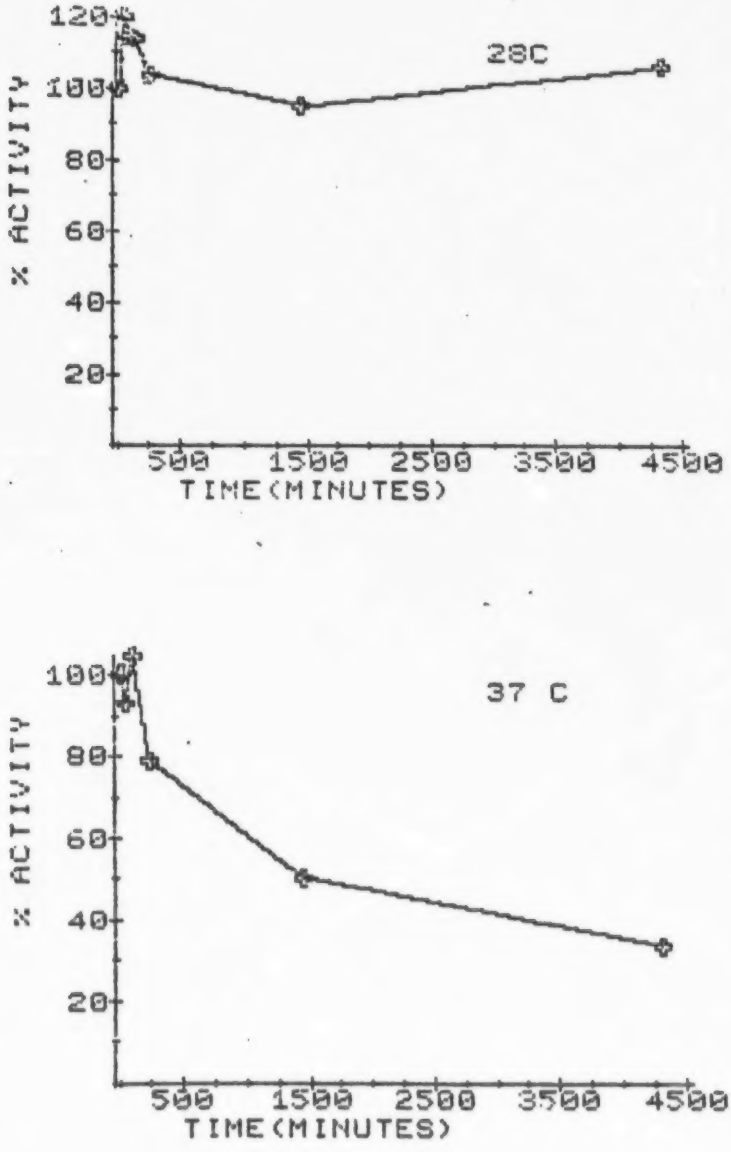
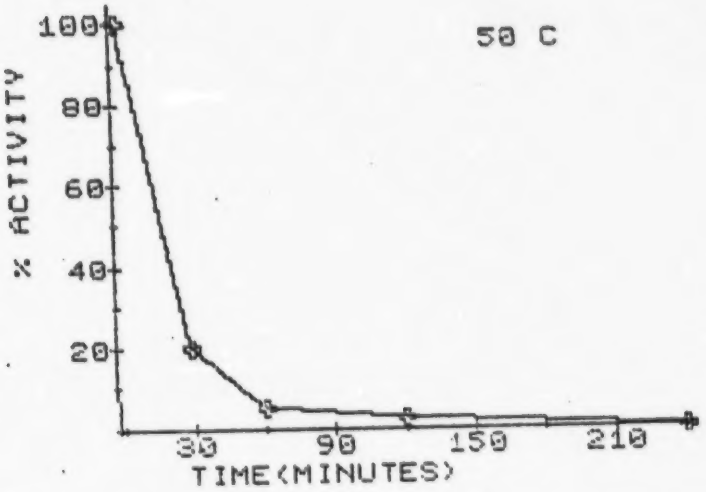


Figure 4b. Thermal stability of endoglucanase activity of the culture filtrate from T. harzianum E58



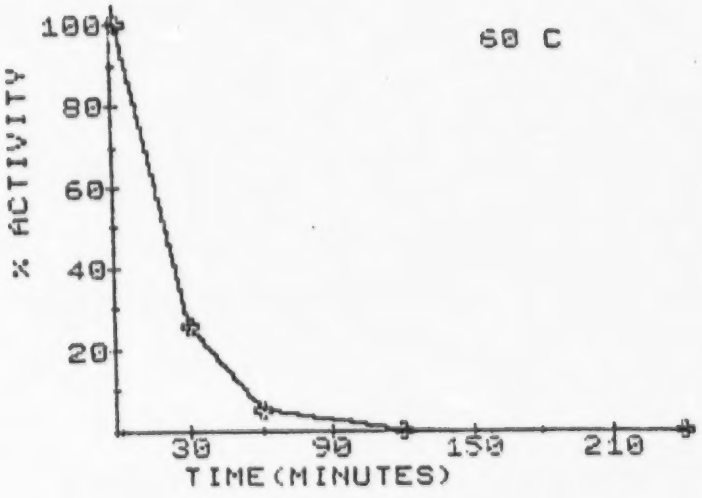
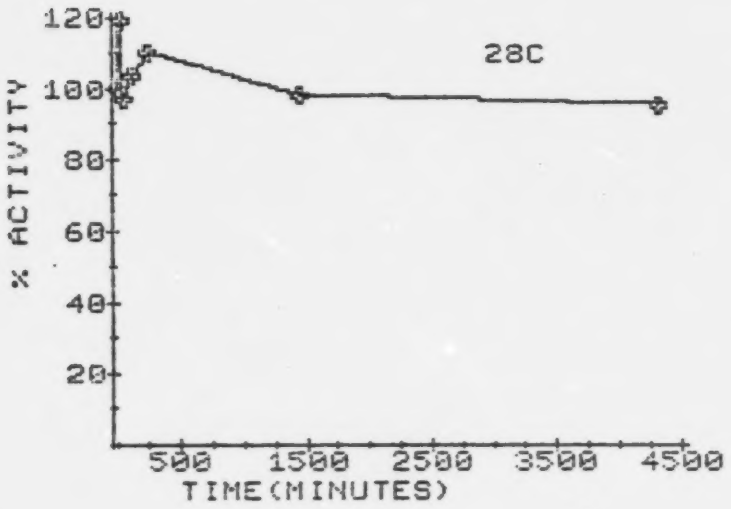


Figure 5a. Thermal stability of β-glucosidase activity of the culture filtrate from T. harzianum E58



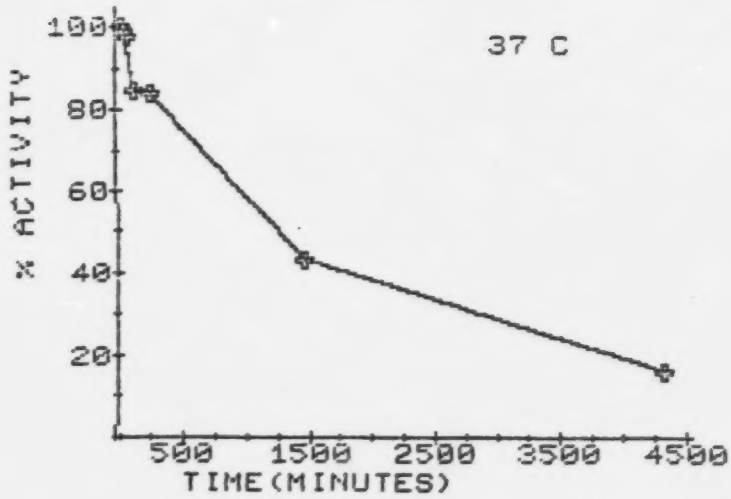


Figure 5b. Thermal stability of β-glucosidase activity of the culture filtrate from T. harzianum E58

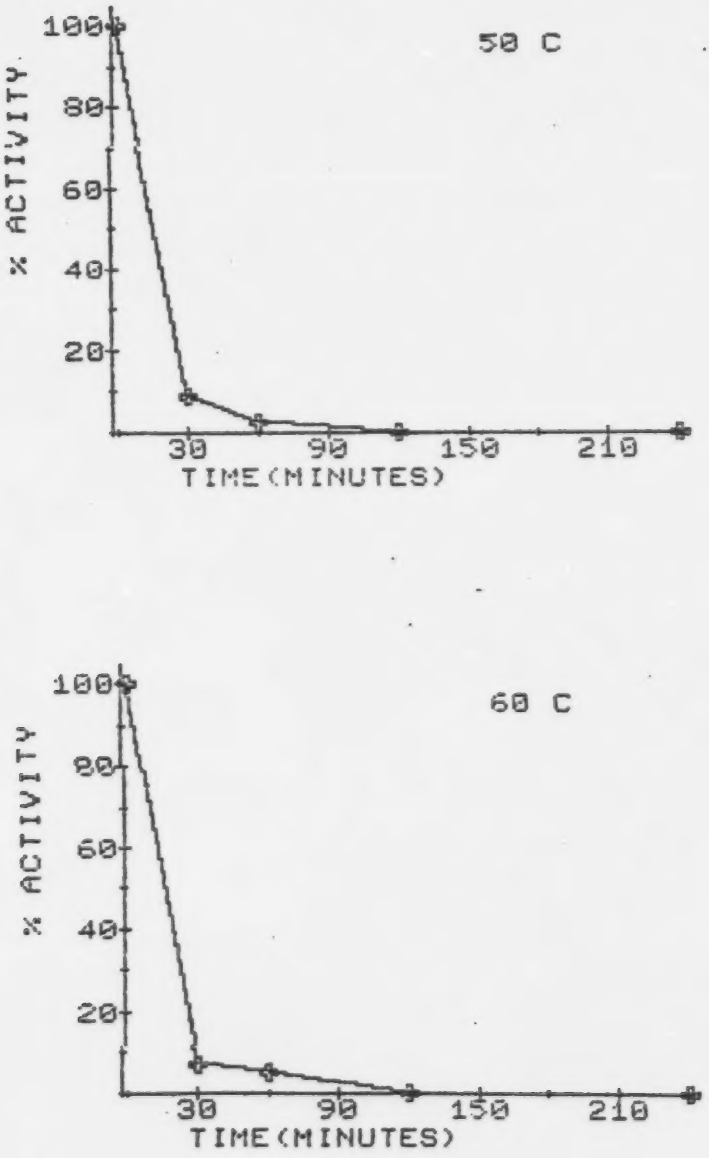
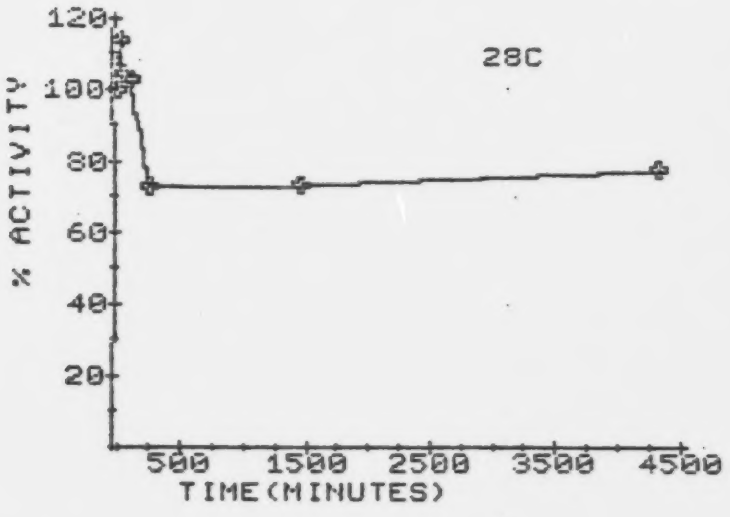


Figure 6a. Thermal stability of filter paper activity of the culture filtrate from T. harzianum E58



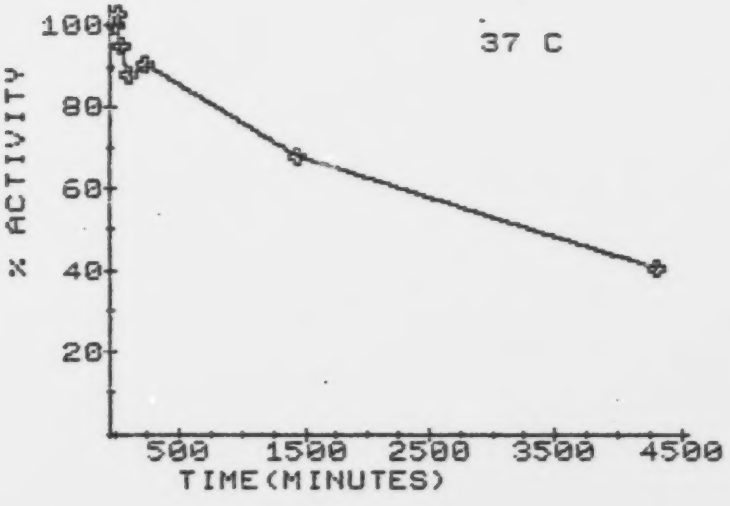
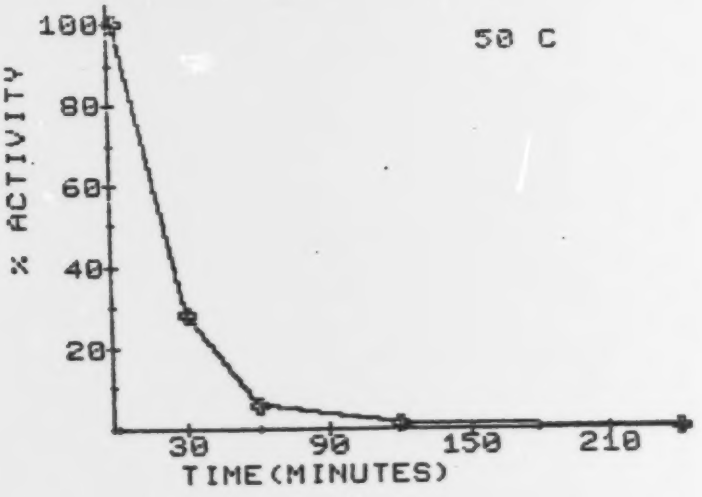


Figure 6b. Thermal stability of filter paper activity of the culture filtrate from T. harzianum E58



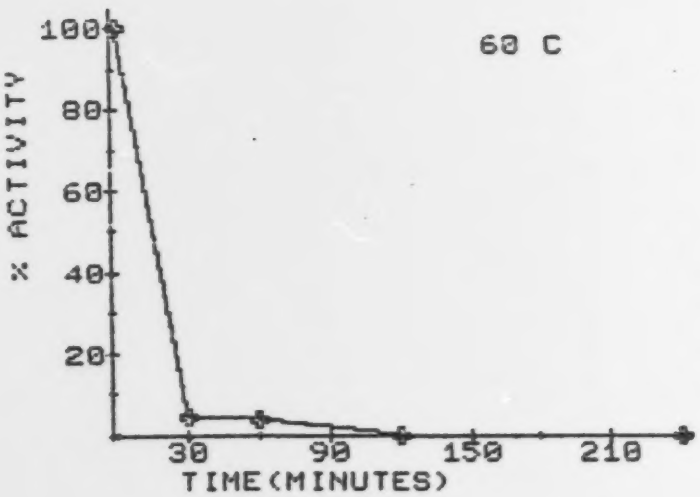
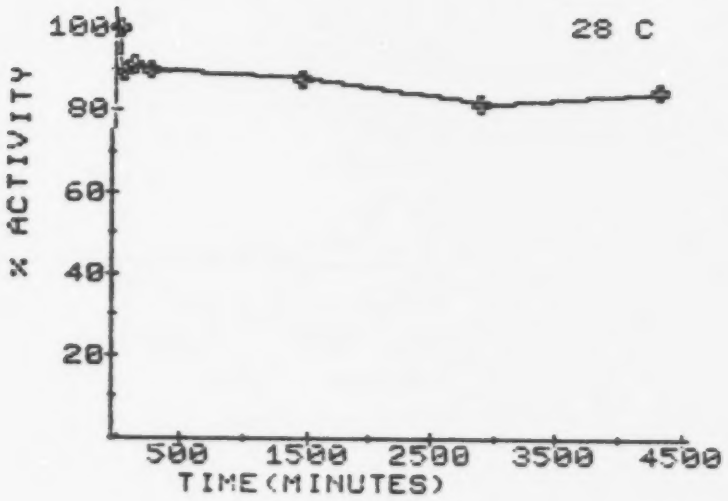


Figure 7a. Thermal stability of endoglucanase activity of the culture filtrate from T. harzianum E58 after filtrate is adjusted to a pH of 4.8



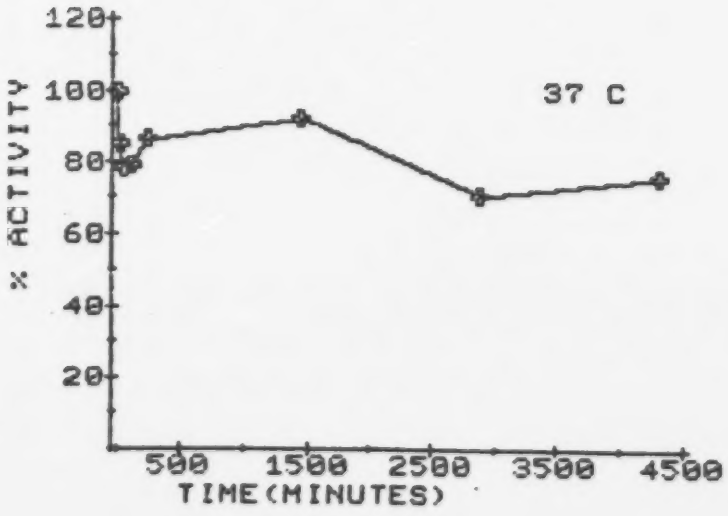


Figure 7b. Thermal stability of endoglucanase activity of the culture filtrate from T. harzianum E58 after filtrate is adjusted to a pH of 4.8

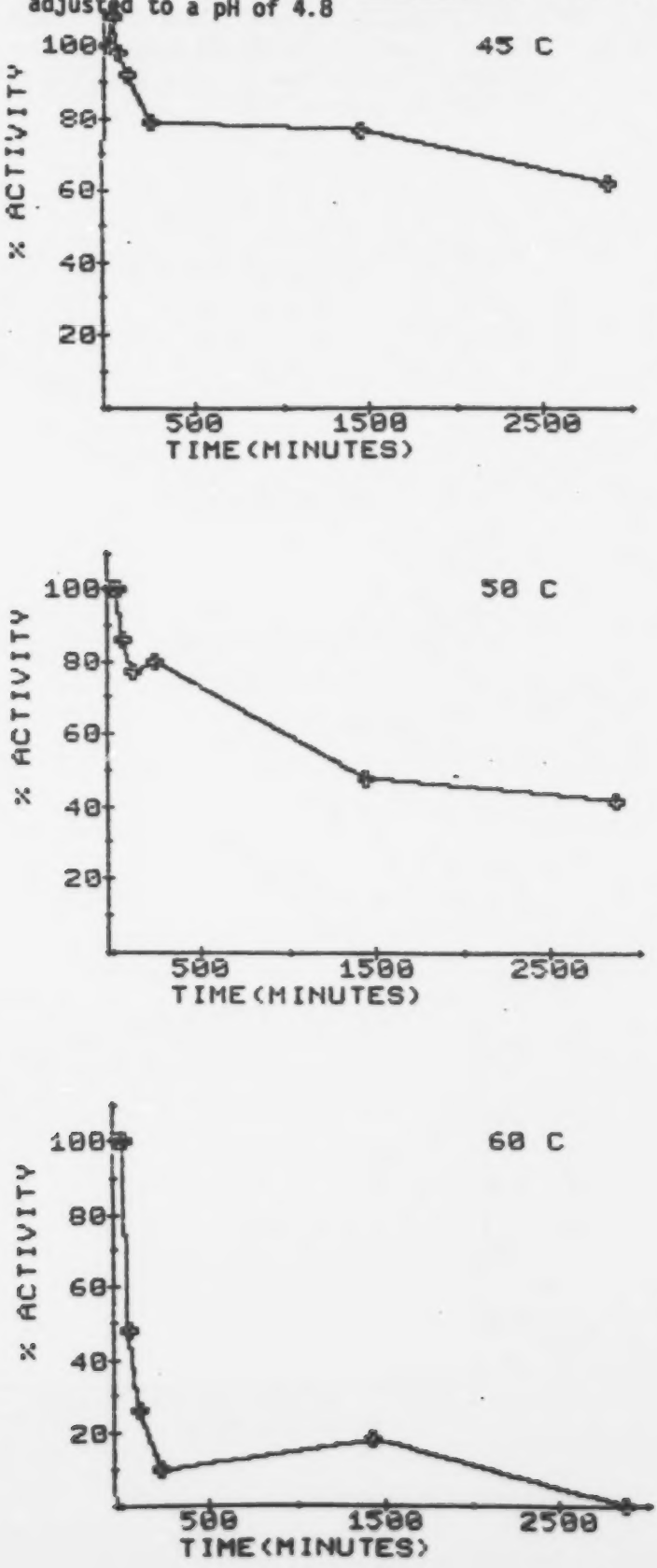
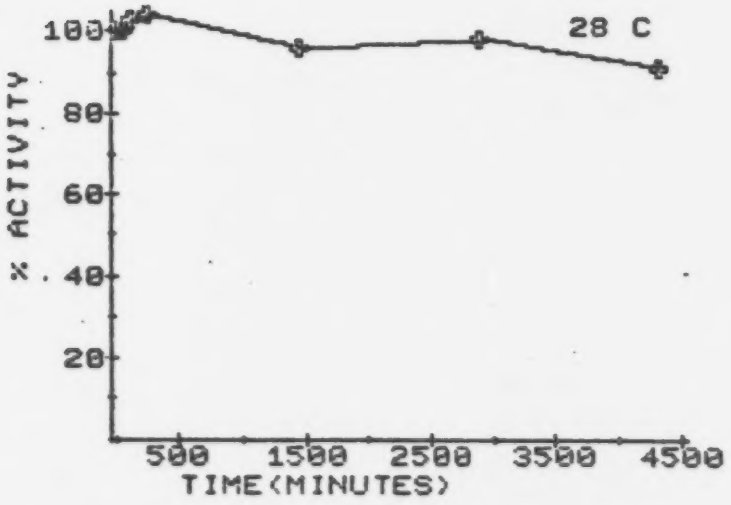


Figure 8a. Thermal stability of β-glucosidase activity of the culture filtrate from T. harzianum E58 after filtrate is adjusted to a pH of 4.8



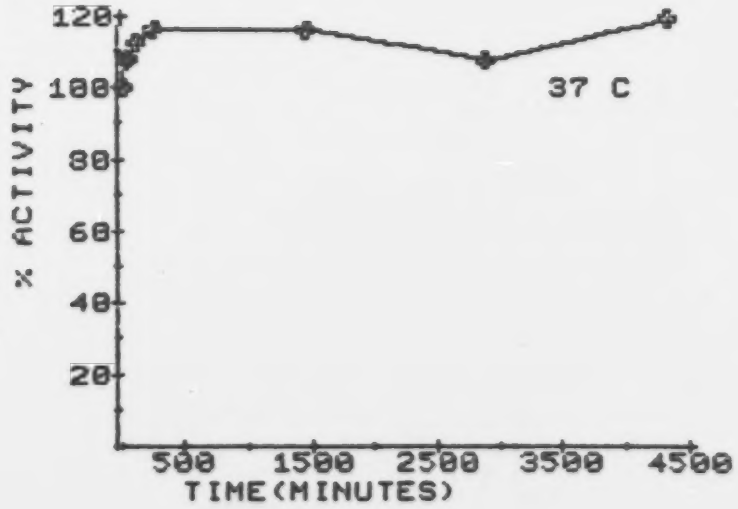


Figure 8b. Thermal stability of β-glucosidase activity of the culture filtrate from T. harzianum E58 after filtrate is adjusted to a pH of 4.8

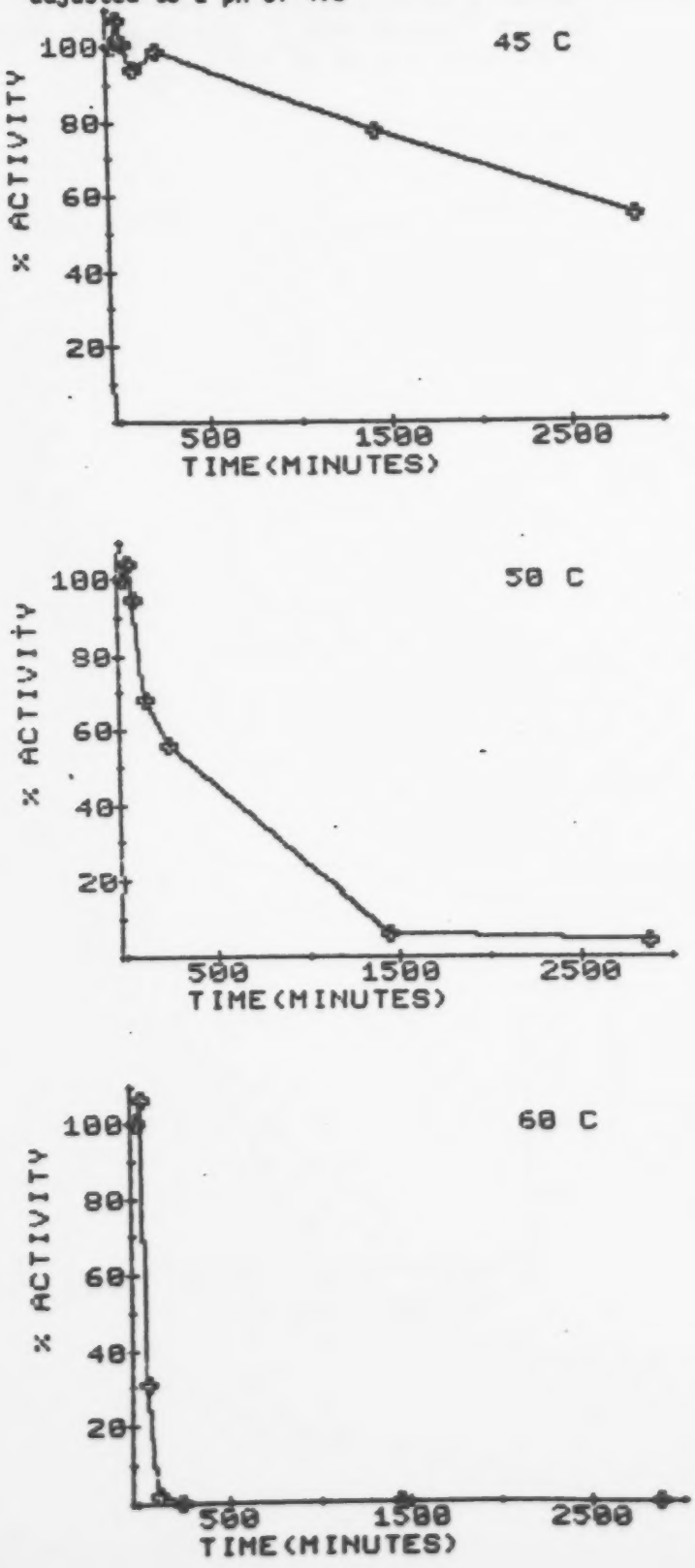
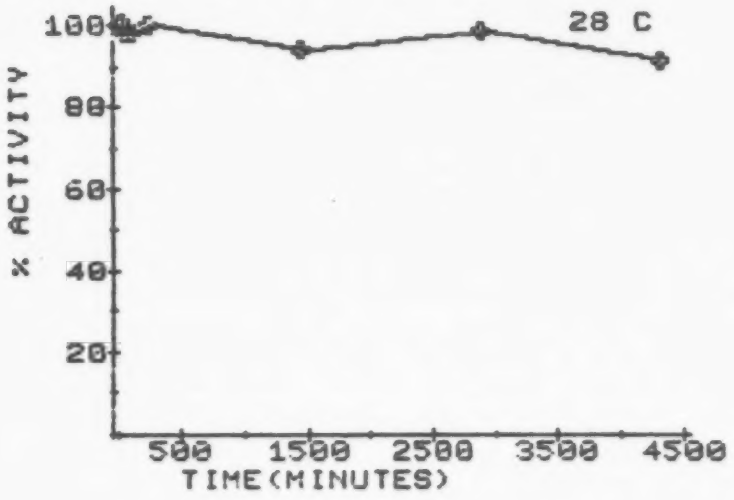


Figure 9a. Thermal stability of filter paper activity of the culture filtrate from T. harzianum E58 after filtrate is adjusted to a pH of 4.8



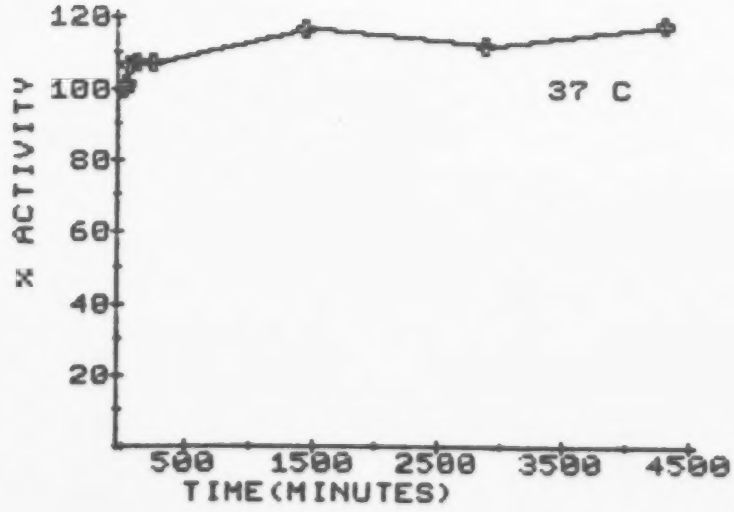


Figure 9b. Thermal stability of filter paper activity of the culture filtrate from T. harzianum E58 after filtrate is adjusted to a pH of 4.8

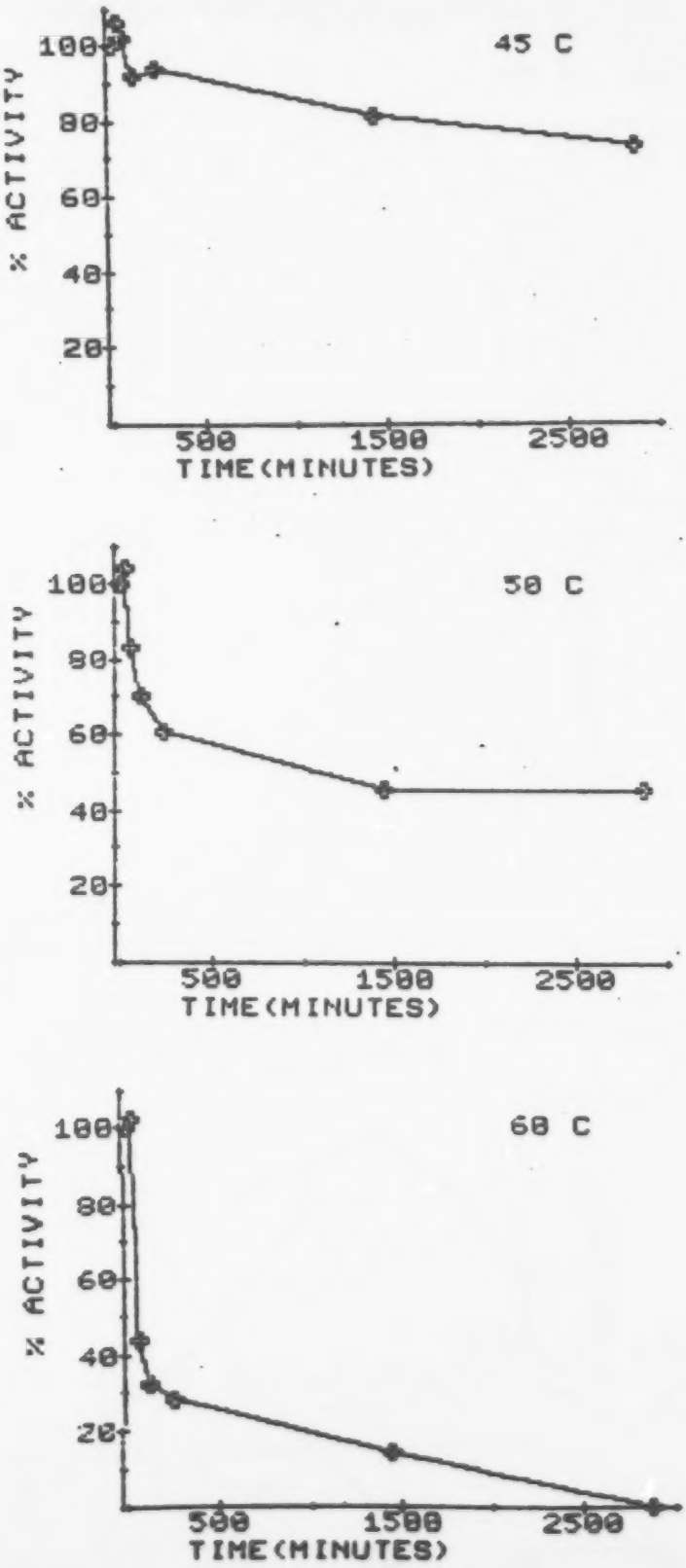


Figure 10. Effect of pH on the activities of endoglucanase (top), ß-glucosidase (middle) and filter paper (bottom) cellulase components of T. harzianum E58. Two buffer systems were employed; pH 4.2-5.6 was achieved using sodium acetate - acetic acid while pH 5.2-6.8 was achieved using citric acid-sodium phosphate.

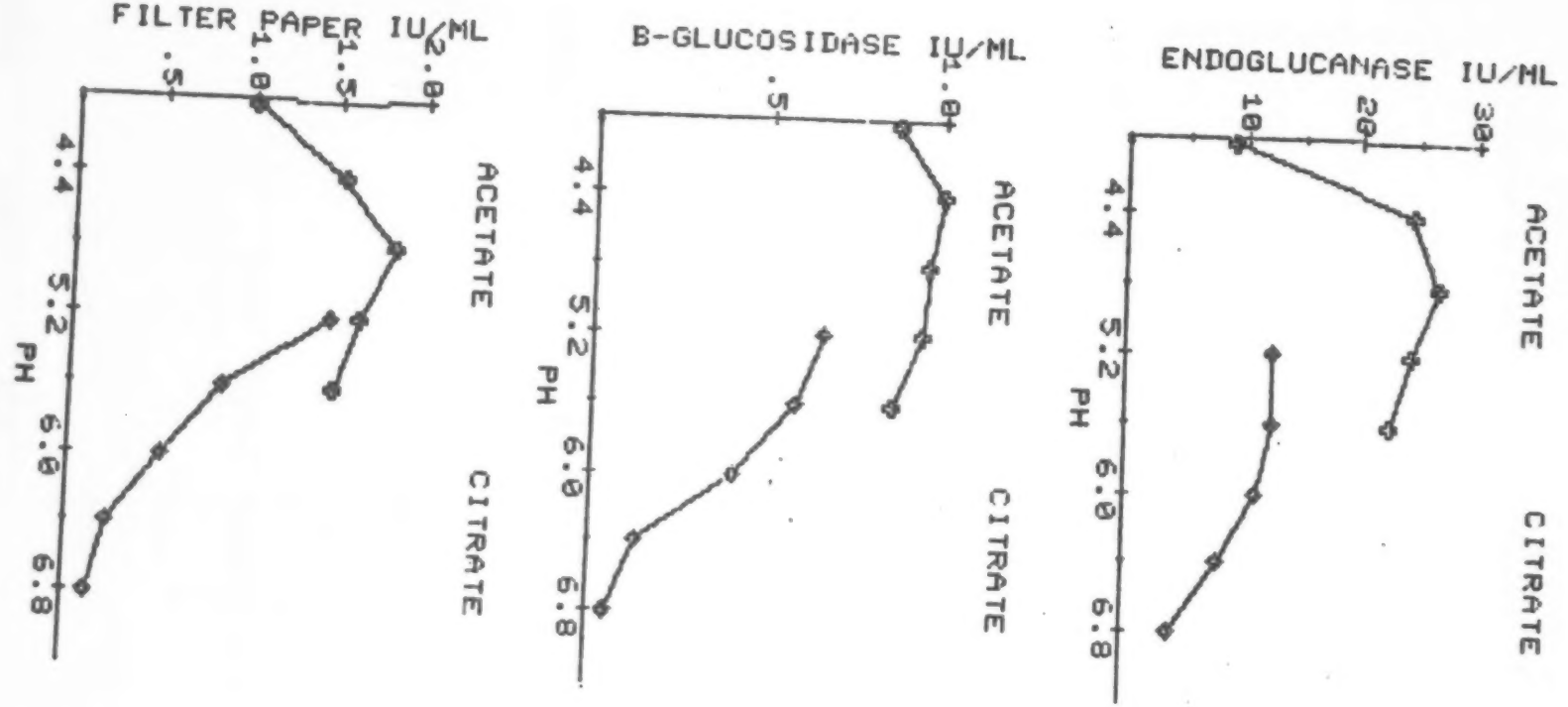


Figure 11. Effect of increasing solka floc concentrations on the production of endoglucanase(top) β-glucosidase (middle) and filter paper (bottom) activities by T. harzianum E58

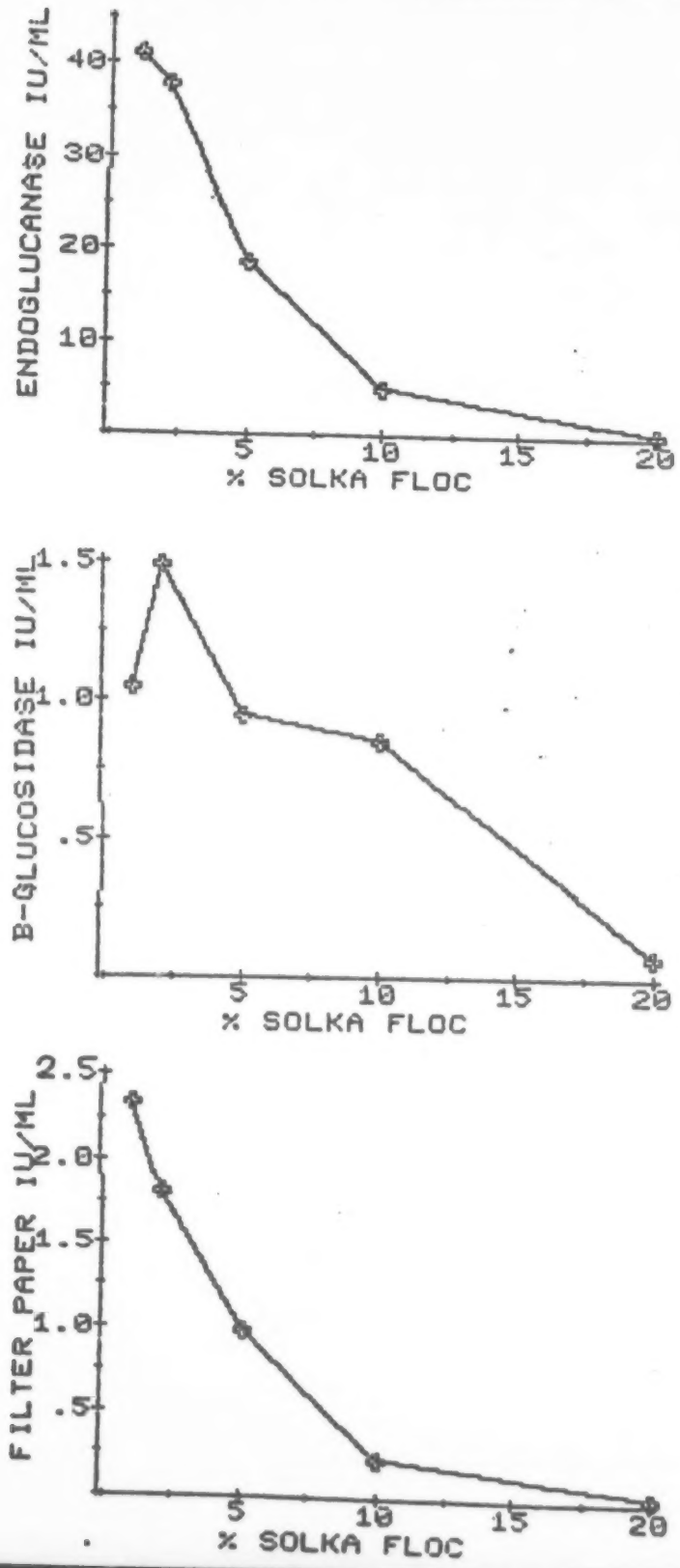
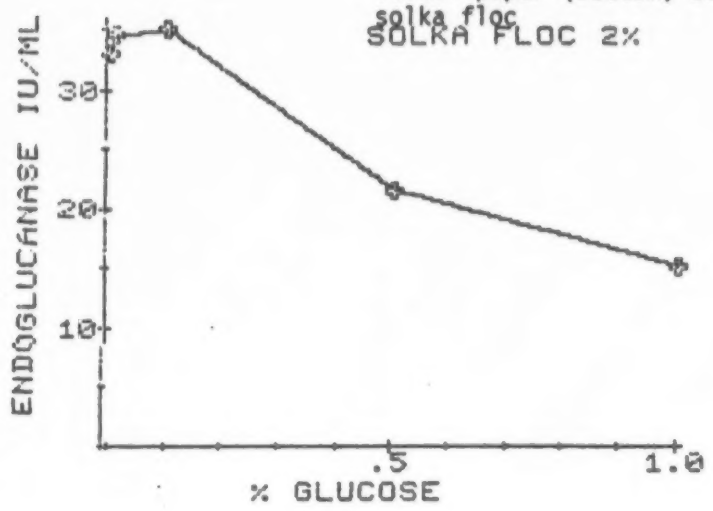
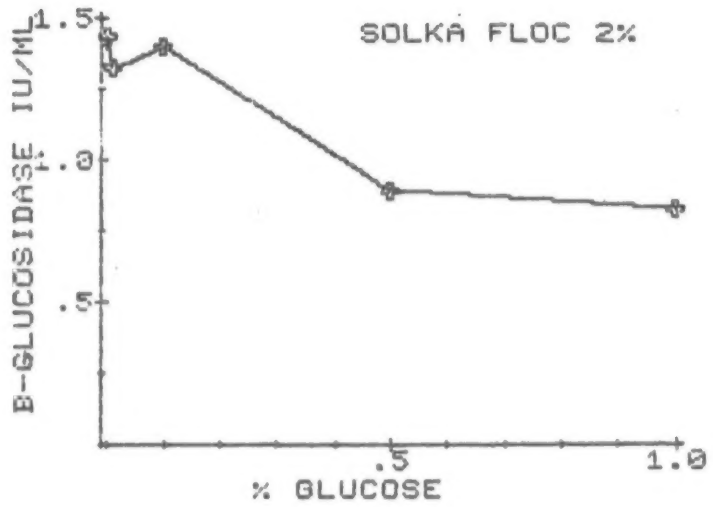


Figure 12. Effect of increasing glucose concentrations on the production of endoglucanase(top), ß-glucosidase (middle) and filter paper (bottom) activities by T. harzianum grown on 2% solka floc





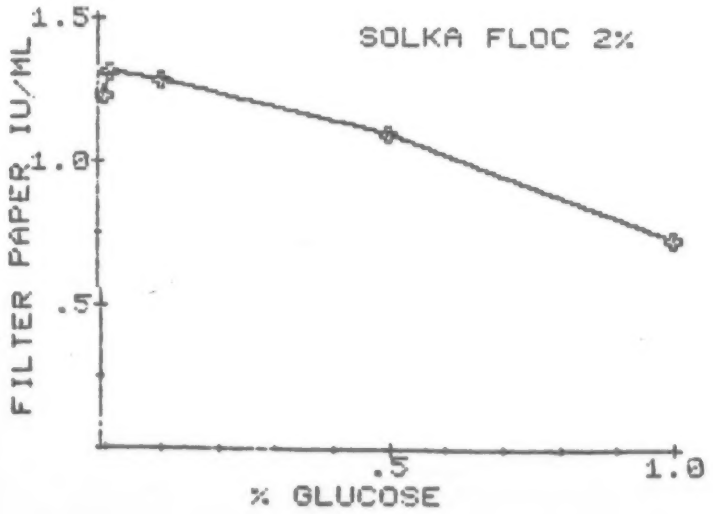


Figure 13. Effect of increasing furfural concentrations on the ethanol production by S. cerevisiae and Z. mobilis grown on 5% glucose.